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**TITLE:** The Use of Comprehensive Molecular Profiling with Network and Control Theory to Better Understand GWI and Model Therapeutic Strategies

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**CONTRACTING ORGANIZATION:** South Florida Veterans Affairs Foundation for Research and Education, Inc.  
Miami, FL 33125

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<b>14. ABSTRACT</b> <p>The objective of this study is to improve our understanding of GWI pathogenesis in two ways through integration across several of the body's regulatory systems of data and knowledge collected from disparate sources, and by mapping of the coordinated interactions between these physiologic systems and the potential for altered "wiring" of these signaling networks in GWI. Using comprehensive molecular profiling, network and control theory the overarching objective of this proposal is to define the precise nature of these irregularities in immune and neuroendocrine signaling as well as the altered activation states of the corresponding cells such that treatment courses can be designed to redirect the system as a whole to normal pattern of coordinated activity.</p> <p>Recruitment is ongoing and as of July 18, 2011, twenty-two patients have been consented to participate in the study; sixteen of the subjects recruited are symptomatic with GWI, four of the subjects are healthy controls and two of the subjects are screen failures. There was a delay in initiating the exercise challenge component of the study due to local requirements, documenting and approvals through several committees on the standard operating procedures for the sterilization of our reusable equipment which consist of a mask attached to the equipment that measures expired gases. This protocol's initial subject assessment was delayed while we attempted to expedite this process. The sterilization process was approved by the Infectious Control Committee and the Reusable Medical Equipment committee as of June 23, 2010 and Exercise Challenges commenced in August 2010. Currently, subjects are being recruited by the use of many different measures. Emphasis has been placed in attracting health Gulf War Veterans during the second half of the annual term.</p> <p>The laboratory measures have been standardized and validated. The analytic plan is being refined, and our co-investigator, Dr Broderick is working with the preliminary data to develop the best method to utilize the data from the preliminary study to validate the findings of the current study. We maintain up to date knowledge of the literature. The PI of this study was tapped to be the Principal Proponent of a large VA cooperative study that hopes to biobank 30,000 GWI era samples, she is very actively engaged in the GWI research and patient advocate community. There is no new literature that would suggest our ongoing study puts research subjects at risk in a newly defined fashion. The study, involving a short exercise challenge and serial blood draws, is a single point in time study, without an intervention. The team is currently working on new viral load measurements, viral survey, and on measures suggested by our genomic studies.</p>				
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## **Introduction**

Within months after their return from Operation Desert Storm an alarming number of Gulf War veterans began to report a variety of symptoms, including fatigue, musculoskeletal discomfort, skin rashes, and cognitive dysfunction. During deployment, these troops were subjected to a number of potentially hazardous conditions and multiple hypotheses as to the etiology of Gulf War Illness (GWI) have been considered. The symptoms of (GWI) that are most consistently reported include those which are often reported in Chronic Fatigue Syndrome (CFS). The objective of this study is to improve our understanding of GWI pathogenesis in two ways; by integration across several of the body's regulatory systems of data and knowledge collected from disparate sources, and by mapping of the coordinated interactions between these physiologic systems and the potential for altered "wiring" of these signaling networks in GWI. Using comprehensive molecular profiling, network and control theory the overarching objective of this proposal is to define the precise nature of these irregularities in immune and neuroendocrine signaling as well as the altered activation states of the corresponding cells such that treatment courses can be designed to redirect the system as a whole to normal pattern of coordinated activity.

## **Body**

The study was approved by the local IRB following the changes that were made, recommended by the DOD, to meet their requirements. Recruitment is ongoing and as of July 18, 2011, twenty-two subjects have been consented to participate in the study; sixteen of the subjects recruited are symptomatic with GWI, four of the subjects are healthy controls and two of the subjects are screen failures. The subjects recruited are between 38-53 years of age. The healthy gulf war veteran recruits consist of three black males and one hispanic male. The gulf war illness recruits consist of 5 white males, 5 black males, 5 hispanic males, and 1 native american female. There was a delay in initiating the exercise challenge component of the study due to local requirements, documenting and approvals through several committees on the standard operating procedures for the sterilization of our reusable equipment, which consist of a mask attached to the equipment that measures expired gases. This mask is reusable, and has to be cleaned in a particular detergent solution. The VA hospital system has recently put into place rigorous standards for documentation of the procedures and adherence to procedures used in sterilizing reusable medical equipment. This protocol's initial subject assessment was delayed while we attempted to expedite this process. The sterilization process was approved by the Infectious Control Committee and the Reusable Medical Equipment committee on June 23, 2010. Since the approval, recruitment of subjects and study procedures has been an ongoing process. Exercise challenge testing was halted in October 2010, due to exercise equipment malfunction and for the completion of maintenance procedures on the equipment used for these test. The cause of equipment malfunction was identified and steps were implemented to prevent this incident from occurring again. Our intentions are to continue recruitment locally regionally and nationally, which entails the increased need for exposure of our study. The creation of a new flyer was accomplished and was approved by our IRB and the VA's Public Affairs Office. The flyer was placed in waiting rooms, on bulletin boards throughout the primary care and specialty clinics located in patient waiting rooms throughout the medical center. Emphasis is being placed on recruiting healthy gulf war veterans during the second half of the annual term. The plan is to collaborate with our local Environmental Health Coordinator, who is responsible for maintaining the local gulf war registry. The goal for the next quarter is to recruit at least 10 healthy gulf war veterans and complete recruitment of the gulf war illness subjects.

As for the collected data, laboratory measures have been standardized and validated. The analytic plan is being refined, and our co-investigator, Dr Broderick is working with the preliminary data to develop the best method to utilize the data from the preliminary study to validate the findings of the current study. We are using several different sorts of analytic methods with this complex data set and are seeing marked differences between GWI and controls, GWI and CFS, with significant differences in homeostatic regulatory balance. Based on our preliminary data, we submitted an intervention study in the 2011 GWI DOD OCMR call for proposals, the LOI was accepted and the protocol submitted in August 2011. We are also seeing abnormalities in the detoxification pathways in the GWI subjects. Our preliminary analysis also shows significant gender differences in the networking and response to exercise. We will be submitting a grant request to delve into these differences. The team is currently working on new viral load measurements, viral surveys, and on measures suggested by our genomic studies. We presented some of the initial results of the overall study at the VA RAC meeting in DC in June 2011. Manuscript preparation is underway.

## **Key Research Accomplishments**

- Established protocol and completed dry run to work out logistics
- Data Management system was established and tested
- Investigators meetings took place between Dr. Nancy Klimas, Dr. Mary Ann Fletcher and Dr. Gordon Broderick in Miami, FL in March 2010 and by conference calls every 2 weeks thereafter
- Research Staff was hired and trained in Spring and Summer of 2010
- Received final approval of the revised ICD and Protocol in April 2010
- Internal VA hurdles were cleared after approval was given June 2010 for a Standard of Practice protocol involving the disinfection of the study's Reusable Medical Equipment
- First subjects were recruited June and July 2010
- Initial visits involving the consenting and screening of the recruited subjects were completed
- Exercise Challenge Test scheduled for all screened subjects beginning August 2010
- Next upcoming Investigators meeting is scheduled for September 2010
- New recruitment methods were proposed and implemented
- Research Advances in GWI and CFS Conference was held on March 26, 2011 at the Miami VA Medical Center. The conference was held to brief Gulf War Veterans on the plans and future of gulf war illness research
- Investigators meetings took place between Dr. Nancy Klimas, Dr. Mary Ann Fletcher and Dr. Gordon Broderick in Miami, FL, March 2011
- Preliminary data presented to the VA GWI RAC in DC June 2011
- Continuation year requested to complete study in June of 2011, study assessments should be complete over the next 5 months, final analysis in spring of 2012.

## **Reportable Outcomes**

We have been using a preliminary data set, supported by a VA Merit, to develop the database and informatics work needed for this work. As the project is still recruiting, it is premature to list publications emanating from this project. The investigators have been developing the analytic platform based on preliminary work and have published 3 papers based on these analyses. Preliminary data was presented at the GWI VA RAC in June of 2011, showing remodeling of regulatory networks and significant differences in metabolic and signaling pathways.

## **Conclusions**

GWI veterans show significant differences in response to exercise when compared to health controls in our preloiminary analysis. There are also gender differences apparent within this relatively small cohort. The study recruitment is still underway, after a year of delay during the initiation of the study while Army/VA IRB issues were sorted out. We are on timeline for concluding this study,a nd its analysis by June of 2012.

Preliminary data is generating early manuscripts and has been used in national meetings, Dr Broderick will be presenting our latest data at the IACFS/GWI meeting in Spetember 2011 (Ottawa, Canada) .

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- 3) Fletcher MA, Zeng XR, Maher K, Levis S, Hurwitz B, Antoni M, Broderick G, Klimas NG. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. *PLoS One.* 2010 May 25;5(5):e10817. PMID: 20520837
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- 5) Fletcher MA, Zeng XR, Barnes Z, Levis S, Klimas NG. Plasma cytokines in women with chronic fatigue syndrome. *J Transl Med.* 2009 Nov 12;7:96. PMID: 19909538
- 6) Broderick G, Fletcher MA, Vernon SD, Klimas N, 2009, Isolating Characteristic Immune Signals under Challenge in Gulf War Illness. Int Assoc CFS/ME, Reno, NV, March 12-15: Oral session Abstracts/ Latest Research in Immunology, abstract #3.
- 7) Broderick G, Andrea Kreitz, Fuite J, Fletcher MA, Vernon SD, Klimas NG. Remodeling of Lymphocyte-cytokine Networks in Gulf War Illness under Challenge. *Brain Behav Imm.* 2010, Under review.
- 8) Broderick G, Fletcher MA, Gallagher M, Vernon SD, Klimas NG. Characteristic Immune Response to Exercise Challenge in Gulf War Illness. *BMC Clin Pathol.* 2009; Under review.

## Appendices

### Appendix A: Journal Publication **Review article**

Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, Klimas N. A pilot study of immune network remodeling under challenge in Gulf War Illness. *Brain Behav Immun.* 2011 Feb;25(2):302-13. Epub 2010 Oct 16.

### Appendix B: Journal Publication **Review article**

Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. Circadian rhythms in cytokine secretion in chronic fatigue syndrome. *Brain Behav Immun.* 2010 Jun 19. [Epub ahead of print] No abstract available. PMID: 20600817

### Appendix C: Journal Publication **Review article**

Fletcher MA, Zeng XR, Maher K, Levis S, Hurwitz B, Antoni M, Broderick G, Klimas NG. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. *PLoS One.* 2010 May 25;5(5):e10817. PMID: 20520837

### Appendix D: Journal Publication **Review article**

Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. A formal analysis of cytokine networks in Chronic Fatigue Syndrome. *Brain Behav Immun.* 2010 May 4. [Epub ahead of print] PMID: 20447453

## **Appendix E: Proceedings**

- 1) Altered immune functions in Gulf War illness and potential therapies  
Dr. Nancy Klimas Miami VA Medical Center,  
June 27, 2011 GWI VA RAC Washington DC
  
- 2) From Cytokines to Cells to Gene Expression: An Integrative Approach to the Study of Gulf War illness Dr. Gordon Broderick University of Alberta  
June 27, 2011 GWI VA RAC Washington DC

## Appendix F: Nancy Klimas, M.D. Curriculum Vitae

### CURRICULUM VITAE

1. Date: July 2010

2. PERSONAL:

2a. Name: Nancy Grace Klimas

2b. Home Phone: (305) 596-5535

2c. Home Address: 10700 S.W. 90th Ave  
Miami, Florida 33176

2d. Citizenship: US

2e. Visa Type: None

2f. Non-Academic Employment: None

2g. Military Service: None

3. ACADEMIC EMPLOYMENT: University of Miami Miller School of Medicine, Department of Medicine

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3b. Office Phone: (305) 575 3267

3c. Current Academic Rank: Professor, tenured

3d. Primary Department: Medicine

3e. Academic Appointments:

1996 - present: Professor of Medicine, University of Miami Miller School of Medicine, Miami, FL (primary)

1997 - present: Professor of Psychology, University of Miami College of Arts and Sciences

1999 - present: Professor of Microbiology and Immunology, University of Miami Miller School of Medicine

1987 - present: Director of AIDS Research, and Co-Director of the AIDS Clinical Research Unit, Miami VA Medical Center

1999 - present: Director, CFS/GWI Multidisciplinary Research Center (initially funded by NIH U01 AI45940)

1985 - present: Co-Director, E.M. Papper Clinical Immunology Laboratory, Division of Rheumatology and Immunology, Department of Medicine, University of Miami School of Medicine

1987 - present: Director, University of Miami Diagnostic Allergy and Immunology Clinic

2000 - present, 1985-1993; Director, VA Allergy Clinic

2001 - 6 month sabbatical, Centers for Disease Control and Prevention, National Center for Infectious Diseases/ Division of Viral and Rickettsial Diseases, Viral Exanthems and Herpesviruses Branch, —~~Hyperic~~ Case Definition of CFS" working group.

1998 - 2000: Director, West Palm Beach VAMC Allergy Clinic

1991 - 1996: Associate Professor of Medicine, University of Miami School of Medicine

1994 - 1999: Associate Professor of Microbiology and Immunology, University of Miami School of Medicine,

1993 - 1997: Associate Professor of Psychology, University of Miami College of Arts and Sciences

1985 - 1991: Instructor and Assistant Professor of Medicine, University of Miami School of Medicine

1986 - Coordinator, Miami VA Medical Center AIDS Program

1984 - National Cancer Institute Research Fellow; Clinical Immunology Lab, Department of Medicine, University of Miami School of Medicine

#### **4. HIGHER EDUCATION**

##### **4a. Institutional:**

Virginia Commonwealth University 9/1972 - 6/1973

University of South Florida, 9/1973 - 6/1976

University of Miami, M.D., 8/1976 - 6/1980

Baylor University Hospitals and Clinics, Medical Internship, 6/1980 – 6/1981

University of Miami Hospitals and Clinics, Medical Residency, 6/1981 – 6/1983

NCI Fellow; Post-Doctoral Fellowship in Diagnostic Laboratory Immunology, E.M. Papper Laboratory of Clinical Immunology, University of Miami, 6/1983 - 6/1984.

#### **5. PROFESSIONAL ACTIVITIES**

##### **5a. Certification, Licensure:**

National Boards Part I, II and III

Florida State Medical License, 1983- current (ME41879)

American Board of Internal Medicine, 1984

ABIM Certification in Diagnostic Laboratory Immunology, 1986

Current hospital affiliations:

Miami VAMC, University of Miami Hospitals and Clinics, University of Miami Hospital, Jackson Memorial Hospital

##### **5b. Publications**

###### **Books and Monographs Published:**

1. Chronic Fatigue Syndrome, P. Goodnik and N.G. Klimas, eds., American Psychiatric Press, Washington, D.C., 1993.
2. Clinical Management of Chronic Fatigue Syndrome, N. Klimas, MD and R Patarca, MD PhD, eds., The Haworth Medical Press, New York • London ,1995
3. Disability and Chronic Fatigue Syndrome. Clinical, Legal and Patient Perspectives. Klimas, N.G., Patarca, R, eds Haworth Press, Inc., New York • London, 1997

###### **Books currently contracted:**

**Chronic Fatigue Syndrome: A Patient's Guide** Johns Hopkins Press; Nancy Klimas, MD

###### **Invited Book Chapters Published:**

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**Article in popular press:**

Klimas, NG Klimas, NG. **Wake-Up Call. Hopeful new research shows that chronic fatigue syndrome may have a genetic basis**. Ms. Magazine, summer issue, 2006.

**INVITED LECTURES (LAST 10 YEARS)**

**Advances in our Understanding of Chronic Fatigue Syndrome**, Pri-Med conference Ft Lauderdale Florida Feb 2010

**Treatment based on pathogenesis – advances in our understanding of CFS**, Fatigue Sciences Conference  
Calgary Canada Sept 2009

Keynote address (opening) **“Immunology of Fatiguing Disorders”** 3<sup>rd</sup> International Conference on Fatigue Science, Okinawa, Japan September 3<sup>rd</sup>, 2008

Keynote Address **“Research Advances in CFS and ME”** Canadian Conference on Fatigue and Illness, Calgary, Alberta Nov 12, 2008

**“Biomarker Discovery in CFS”** 8<sup>th</sup> annual TMJ Conference Rockville, MD, May 12, 2008

**“Research Advances in CFS/ME”** Invited lecture Western Pharmacologic Association Kona, Hawaii Jan 29, 2008

Keynote address: **Understanding the interactions of the Immune system and the brain in CFS;** International CFS/ME Clinical Conference, Oslo, Norway October 8, 2007

**Evidence Based Treatment Approaches in CFS/ME; International CFS/ME Clinical Conference, Oslo, Norway October 8, 2007**

**Advances in our understanding of the immunology of CFS** Keynote 2<sup>nd</sup> International Conference on ME/CFS Biomedical Research, May 25, 2007 Edinburgh, Scotland.

**Diagnosis and Management of CFS**, Feb 23, 2007 PRIMCARE conference Ft Lauderdale FL,

**Research Updates, ME/CFS** February, 2007, Madrid and Barcelona, Spain, Universad de Catilonia, series of talks to clinicians and patients —

**Presidential Address, "New Directions in CFS/ME Research"** IACFS/ME 8<sup>th</sup> annual International Research and Clinical meeting, Ft. Lauderdale, FL January 12, 2007.

**The Diagnosis and Management of ME/CFS**, a series of 10 talks to professionals and patients, across the country September, 2006, New Zealand

**The Immunology and Genomics of Gulf War Illness** – August 14, 2006 GWI Research Advisory Council, Washington, DC

**Research Methodology in Fatiguing Illnesses** Keynote Address Aviano, Italy: 1st International Meeting on Chronic Fatigue Syndrome and Cancer-related Fatigue\_May 5th 2006

**Chronic Fatigue Syndrome: From Genomics to Treatment:** Keynote Address, Connecticut ME Association Regional Conference, April 30, 2006 Hartford CT

**CFS In the Veteran Population:** Best Practices in the Continuum of Care: Management of Infectious Disease Little Rock AR.April 26, 2006

**Clinical Management of CFS** - PANDORA Conference, West Palm Beach, FL Oct 27, 2005

**Research Advances in CFS – Keynote Address** , OFFER Regional Conference, Salt Lake City ,Utah 4/16/05

**Impact of Research Advances on Clinical Management of CFS** – Keynote, OFFER Patient Conference, Salt Lake City, Utah 4/16/05

**Research Advances in CFS – Keynote Address** , CFIDS Asso Regional Conference, Charlotte, NC Nov 13, 2004

**Gene Array Technology in CFS** – the C<sup>3</sup> Computational Challenge, Cold Spring Harbor, Oct 2005

**Immunomodulatory therapies – a review**, South Florida Allergy Journal Club, 11/04

**CFS – advancing knowledge impact on management, Keynote, AACFS Intl Clinical Conference, Madison Wisconsin 10/04**

**CFS Pathogenesis Keynote address,** Specialisation Course on Fibromialgia and Chronic Fatigue Syndrome International University of Catalonia Barcelona, Spain May 29, 2004. Honorary degree awarded.

**Management of CFS Keynote Address,** PANDORA Providers and Patient Conference, Ft Lauderdale FL May 11, 2004

**The Diagnosis and Management of CFS** NMA 2003 Annual Convention and Scientific Assembly, Philadelphia, August 2003

**Immune Methodologic Issues,** invited speaker NIH CFS Methodology Workshop June 2003, Bethesda, MD

**CFS and Fibromyalgia – Diagnosis and Management** NPACE, May 2003 Orlando FL

**CFS: What we know, what we need to know, and how to get there.** New Jersey Medical Association, New Brunswick. May 2003

**CFS: What we know, what we need to know, and how to get there.**, Regional Primary Care Conference Salt Lake City May 2003

**CFS – Somatic or Physical? A Debate,** Intl Behavioral Medicine Asso, Helsinki, Finland August 2002

**Instruments and Design of an Empiric Case Definition Study,** CDC CFS Case Definition Workshop, Calloway Gardens, May 2002

**Diagnosis and Management of CFS,** National American Medical Women's Association Conference, San Antonio, TX Jan 2002

**Inclusion and Exclusion Criteria,** CDC CFS Case Definition Workshop, Calloway Gardens, May 2001

**Immunology of Chronic Fatigue Syndrome,** State of the Science Meeting, NIH, October 2000

**Current Understanding of CFS Pathogenesis,** NIH/CFS Research Priorities Series – Autonomic Dysfunction Conference, October 2000; Neuroendocrine Abnormalities, February 2001; Immunology Conference October 2001

**Immune Restoration Post Antiretroviral Therapy,** Guest lecturer, Hollywood Memorial Hospital; Baptist Hospital; Mercy Hospital; Broward General Hospital; VAMC, Nashville Tennessee; Mobile, Alabama; Key West Florida; Jan – April 2005

**Global Impact of HIV on Women,** National Organization for Women's Global Women's Health Conference, Washington DC June 2001

**Housestaff lectures** (given regularly throughout all academic years): Anaphylaxis; Asthma; Immune Modulatory Therapies; Global Impact of HIV; Immune restoration in HIV infection; Hepatitis C; Hepatitis C; HIV Co-infection; Death and Dying – the Clinician's role; Chronic Fatigue syndrome; Gulf War Syndrome; Psychoneuroimmunology; Stress and Disease.

**Graduate Studies Lectures:** Multidisciplinary Clinical Research; Immunology 101; Psychoneuroimmunology and disease; Pathogenesis of HIV/AIDS; Chronic Fatigue Syndrome

**Undergraduate Lectures:** Careers in clinical research, Death and Dying

## **A. Other Works and Publications**

### Letters to the Editor

1. Klimas, N.G. and Fletcher, M.A. Chronic Fatigue Syndrome. JAMA, 1991.

2. Klimas, N.G., Blaney, N., Morgan, R. and Fletcher, M.A. Methadone and Immune Function, Amer. J. Med. 92:114-115, 1991.

### Editorials

1. Klimas, N.G. and Patarca, R. The birth of a journal. J. Chronic Fatigue Syndrome. 1:1-2, 1995.

### Abstracts and Presentations at National and International Meetings

1. Klimas, N.G., Lo, H., Brunschwig, J.P., Caldwell, K.E., Latif, Z.A., and Fletcher, M.A. Biochemical Morphological and Receptor Properties of Erythrocyte Glycoproteins, Soc. Complex Carbohydrates, 1980.
2. Klimas, N.G., Caldwell, K.E., Latif, Z.A. and Fletcher, M.A.: A Purified Erythrocyte Bovine Glycoprotein Useful In The Serodiagnosis of Infectious Mononucleosis. Clin. Res. 29 No. 5, 1981.
3. Scott, G.B., Parks, W.P., Fischl, M., Fletcher, M.A. and Klimas, N.G.: Acquired Immunodeficiency Syndrome in Haitians: A Household Approach. Presented to the Cold Spring Harbor Special AIDS Meeting. Cold Spring Harbor, New York, September 17, 1983.
4. Klimas, NG. and Fletcher, M.A: Elevation of "Forssman-like" Antibody in AIDS. Clin. Res. 42:350, 1984.
5. Fischl, M.A., Klimas, N. G., Wang, G., Georgiades, J.A., Spira, T. and Fletcher, M.A. Human -lymphoblastoid Interferon in the Treatment of Kaposi's Sarcoma, Internatl. Interferon Conf., Frankfurt, 1984.
6. Baron, G.A., Klimas, N.G., Fischl, M. and Fletcher, M.A.: Decreased Natural Cell Mediated Cytotoxicity (NCC) to Cell-Line K562 per Leu 11 Positive Cells in Acquired Immunodeficiency Syndrome (AIDS). Fed. Proc. 44:593,1985.
7. Fischl, M.A., Dickinson, G., Scott, G., Klimas, M.A., Fletcher, M.A. and Parks, W.: Evaluation of Household Contacts of Adult Patients with the Acquired Immunodeficiency Syndrome, 1st Internatl. Conf. on AIDS, Atlanta, 1985.
8. Fischl, M.A., Ahn, Y.S., Klimas, N.G., Harrington, W.J. and Fletcher, M.A.: Use of Danozol in Autoimmune Thrombocytopenia Associated with the Acquired Immunodeficiency Syndrome. 1st Internatl. Conf. on AIDS, Atlanta, 1985.
9. Baron, G.A., Klimas, N.G., Fischl, M.A. and Fletcher, M.A.: Natural Cell Mediated Cytotoxicity (NCC) to Cell Line K562 per Leu 11 Positive Cell is Decreased in the Acquired Immunodeficiency Syndrome. 1st Internatl. Conf. on AIDS, Atlanta, 1985.
10. Klimas, N.G., Torres, O., Silveira, G., Fischl, M.A. and Fletcher, M.A.: Humoral Immune Abnormalities in Two South Florida Acquired Immunodeficiency Syndrome Risk Groups. 1st Internatl. Conf. on AIDS, Atlanta, 1985.
11. Scott, G.B., Fischl, M.A., Klimas, N.G., Fletcher, M.A., Dickinson, G. and Parks, W.: Mothers of Infants with the Acquired Immunodeficiency Syndrome (AIDS) : Outcome of Subsequent Pregnancies. 1st Internatl. Conf. on AIDS, Atlanta, 1985.
12. Klimas, N.G., Lian, E., Fischl, M.A. and Fletcher, M.A.: Apparent False Positive ELISA Tests for HTLV/LAV Antibody (AB) and Polyclonal B cell Activation (PCBA) in Acquired Immunodeficiency Syndrome (AIDS) Risk Groups. 2nd Internatl. Conf. on AIDS, Paris, 1986.
13. Fischl, M.A., Dickinson, G.M., Scott, G.B., Klimas, N.G., Fletcher M.A. and Parks, W.: Heterosexual and Household Transmission of the Human T-lymphotropic Virus Type III. 2nd Internatl. Conf. on AIDS, Paris, 1986.
14. Klimas, MA., Lubs, M. and Fletcher, M.A. Complement (C') Activation and Immune Complex (IC Formation in Asymptomatic Women with Multiple Miscarriages. 6th International Congress of Immunology, Toronto, 1986.
15. Fletcher, M.A. and Klimas, N.G. Polyclonal B cell activation (PBA) and the Incidence of Antibody to HTLVIII/LAV (AB) in Groups at Risk for Acquired Immunodeficiency Syndrome (AIDS). 6th International Congress of Immunology, Toronto, 1986.
16. Baron, G.C., Klimas, N.G., Ashman, M.A., Fischl, M.A. and Fletcher, M.A.: Immune Parameters of Patients with AIDS/Kaposi's Sarcoma During Human Lymphoblastoid Interferon Treatment. 3rd International Congress on AIDS, Washington, D.C., 1987.

17. Dodds, S., Fletcher, M.A., P.O'Hearn, Cole, P., Caralis, P., Klimas, N.G., et al.; Inter- and Intra- Personal Factors in the Recruitment and Retention of Subjects For an AIDS Intervention Research Study. 3rd International Congress on AIDS, Washington, D.C., 1987.

18. Baron, G.C., Bouguignon, L.Y.W., Klimas, N.G., Fischl, M.A., Scott, G.B. and Fletcher, M.A.: Reduced Lymphocyte Cap Formation in Patients with AIDS and ARC. 3rd International Congress on AIDS, Washington, D.C., 1987.

19. Tarsis, S.L., Klimas, N.G., Baron, G.S., Ashman, M.A. and Fletcher, M.A.: Decreased Natural Killer Cell Activity and Changes in T Lymphocyte Surface Markers in Patients with Chronic Epstein Barr Virus (EBV Infection. 5th Annual Medical Laboratory Immunology Conference, Williamsburg, VA, 1987.

20. Eisdorfer, C., Szapocznik, G. Scott, Fletcher, M.A., Klimas, N.G., Fordyce-Baum, M. The Biopsychosocial Research Center on AIDS: A Multidisciplinary Approach to the Investigation of the AIDS Disease. 3rd International Congress on AIDS, Washington, D.C., 1987.

21. Fletcher, M.A., Caralis, P., Laperrier, A., Ironson, G., Klimas, N.G., Perry, A., Ashman, M. and Schneiderman, N. Immune Function and Aerobic Training as a Function of Anti-HIV Status in Healthy Gay Males, 4th International Conference on AIDS, Stockholm, Sweden, June, 1988.

22. Fletcher, M.A., O'Hearn, P., Ingram, F., Ironson, G., Laperrier, A., Klimas, N.G. and Schneiderman, N. Anticipation and Reaction to Anti-HIV Test Results: Effect on Immune Function in an AIDS Risk Group. 4th International Conference on AIDS, June, 1988.

23. Fletcher, M.A., Ironson, G., Caralis, P., Laperrier, A., Klimas, N.G., O'Hearn, P., Antoni, M. and Schneiderman, N. A Behavioral Intervention Study in Anti-HIV Positive and Negative Gay Men. Presented, American Psychological Society, August, 1988.

24. Laperrier, A., O'Hearn, P., Ironson, G., Caralis, P. Ingram, F., Perry, A., Klimas, N.G., Schneiderman, N. and Fletcher, M.A. Exercise and Immune Function in Healthy HIV Antibody Negative and Positive Gay Males. 9th Annual Society of Behavioral Medicine. Boston, MA, 1988.

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26. Klimas, N.G., Page, B., Chitwood, D., Ashman, M. and Fletcher, M.A. Immune Abnormalities in Street Intravenous Drug Users. 4th International AIDS Conf. Stockholm, 1988.

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28. Klimas, N.G., N. Blaney, Morgan, R., Ashman, M. and Fletcher, M.A. Abnormal Lymphocyte Subsets and Natural Killer Cell Function in Anti-HIV Negative Intravenous Drug Users on Methadone. 4th International AIDS Conf., Stockholm, June, 1988.

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30. Klimas, N.G. The Psychoimmunology of HIV Infection in IV Drug Users. Presented at the American Psychological Association, Atlanta, 1988.

31. Millon, C., Salvato, F., van Riel, F., Blaney, N., Fletcher, M.A. and Klimas, N.G. Psychosocial Characterization of Chronic Fatigue Syndrome/Epstein-Barr Virus Patients. *J. Exp. Clin. Can. Res.* 7, #3, supplement:87, 1988.

32. Van Riel, F., Mantero-Atienza, E. Salvato, F., Beach, R., Fletcher, M.A., Klimas, N.G. and Fordyce-Baum, M. Nutritional Status of Chronic Fatigue Syndrome (CFS)/ Epstein-Barr Virus (EBV) Patients. *J. Clin. Can. Res.* 7, #3, supplement: 88, 1988.

33. Salvato, F., Fletcher, M.A., Ashman, M. and Klimas, N.G. Immune Dysfunction Among Chronic Fatigue Syndrome (CFS) Patients with Clear Evidence for Epstein-Barr Virus (EBV) Reactivation. *J. Clin. Can. Res.* 7, #3, supplement: 89, 1988.

34. Laperriere, A., Ironson, G., Klimas, N., Caralis, P., Antoni, M., Fletcher, M.A. and Schneiderman, N., Aerobic Exercise and Immune Function in an AIDS Risk Group. presented at a symposium at the Society of Behavioral Medicine, San Francisco, CA, 1989.

35. Laperriere, A. Ironson, G., O'Hearn, P., Caralis, P., Antoni, M., Ingram, F., Klimas, N., Baggett, L., Schneiderman, N. and Fletcher, M.A. Aerobic Exercise Training as a Buffer of Anxiety and Depression in HIV Infected Individuals. Soc. Behav. Med., Boston, 1989.

36. Klimas, N.G., Blaney, N., Morgan, R., Simoneau, J. and Fletcher, M.A. Immunologic Abnormalities in Anti-HIV-1 negative (-) and positive (+) IV drug Abusers (IVDA) on Methadone. V Internatl. Conf on AIDS. Montreal, 1989.

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46. Fletcher, M.A., Klimas, N.G., Salvato, F., Millon, C. and Ateinza-Mantero, E. Immunological Parameters of Chronic Fatigue Syndrome (CFS). VII Internatl. Cong of Immunology, Berlin, 1989.

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51. Klimas, N.G. Epidemiology, Clinical Manifestations and Therapy of HIV-1 Infections. Symposia International Sobre SIDA-1990, Buenos Aires, Argentina, 1990.

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54. Pando, J. and Klimas, N.G. Clinical vs Necropsy Findings in an AIDS Population. Sixth International Conference on AIDS, San Francisco, CA, 1990.

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93. Maher K, Asthana D, Patarca R, Klimas NG, and Fletcher MA. CD69 on CD4 and CD8 Cells From HIV-1 Infected Donors Clinical Applications of Cytometry. Charleston, SC, 1995.

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95. Maher K, Klimas N, Dickinson G, Triplett J, Maggio C, Cohen V, Valenzuela R, and Fletcher MA. The effect of Highly Active AntiRetroviral Therapy on Viral Load and the Immune System in Human Immunodeficiency Virus (HIV) Infection. NY State Wadsworth Conference on HIV Laboratory Testing. Albany, NY, 1997.

96. Maher, K, Klimas, N, Dickinson, G, Triplett, J, Maggio, C, Cohen, V, Valenzuela, R and Fletcher, MA. T-lymphocyte subsets and highly active anti-retroviral therapy (HAART) therapy of human immunodeficiency virus (HIV) infection, selected for oral presentation. Clinical Applications of Cytometry. Charleston, SC, 1997.

97. Klimas, N. Update on anti-retroviral therapy in HIV. SCIM, Vail Colo., 1997.

98. Klimas, N. Highly active anti-retroviral therapy: implications for adherence and immunomodulatory interventions. Academy for Behavioral Medicine Research, Cape Cod, 1998.

99. McPherson, S., Malow, R., Penedo, F.J., Jones, D., Maggio, C., Triplett, J., Klimas, N., Enhancing adherence to combination antiretroviral therapy in non-adherent HIV+ men. Presented at the annual meeting of the American Psychological Association, Boston, MA, 1999.

100. Klimas, NG - Immunology of CFS, Regional Behavioral Medicine Conference, Auckland New Zealand, Nov 2000

101. Klimas, NG, McPherson, S Adherence and Chronic Active Hepatitis C – what we have learned from HIV, 25<sup>th</sup> Intl behavioral Medicine Society Conference, Brisbane, Australia Nov 2000

102. Immunology of CFS, State of the Science Meeting, NIH Workshop, Washington DC October 2000

103 Klimas, NG , speaker: Current understanding of the Pathogenesis of CFS – NIH/CFIDS Asso Workshop on Autonomic Dysfunction and CFS, February 2001, Washington DC

104 Klimas, NG , speaker: Methodologic Issues Seminar \_ NIH/CFIDS Workshop on Neuroendocrine Dysfunction and CFS, Nov 2001, Washington DC

105 Klimas, NG , speaker: Methodologic Issues in the Study of CFS – NIH/CFIDS Association Workshop on Immune Dysfunction and CFS, Feb 2002

106 Klimas, NG , speaker: Adherence to Therapy Seminar, 26<sup>th</sup> International Behavioral Medicine Society Conference, Helsinki, Finland, August 2002

107 Klimas, NG, debater participant: CFS – physical or somatic disorder? 26<sup>th</sup> International Behavioral Medicine Society Conference, Helsinki, Finland, August 2002

108 Klimas, NG , speaker : Methodologic Issues – Immunology of CFS; NIH State of the Science Meeting, Bethesda, MD, May 2003

109 Klimas NG and Turgeli, E Assessment Tools in CFS, AACFS Intl Conference Madison Wisconsin, October 2004

110 Jeffrey Greeson, Maria Llabre, Nancy Klimas, Peter Lawrence, Alex Gonzalez, Pedro Martin, Neil Schneiderman, Barry Hurwitz Psychological Distress and HIV Disease Progression: Roe of Natural Killer Cell Immunity; 2005 Annual Meeting of the American Psychosomatic Society, March 2-5, in Vancouver, Canada

111 Elevations of HHV-6 serology are associated with low NK cell Function , Nancy Klimas, Mary Ann Fletcher, Kevin Maher. International Conference on HHV6 Infection, Barcelona Spain May1-3, 2006.

112 Klimas, NG and Fletcher MA Neuropeptide Y in CFS and GWI, IACFS International Conference, Fort Lauderdale 2007

113 Vera, M, Klimas, N, Garcia L,, Fletcher MA Isoprinosine in CFS (presentation) IACFS/ME Research Conference Reno Nevada, March 2009

114 Garcia, L, Klimas N, Fletcher MA Incidence of sleep disorders in CFS sample. IACFS/ME Research Conference Reno Nevada March 2009

5c. **Funded Research (Past 5 years):**

**Pending:**

**Cooperative studies program, Principal Proponent: Assessing genetic variables in gulf war illness, LOI accepted, protocol development funded.** March 2010

**"Genetic pathway Analysis in CFS"** – NIH protocol submitted Mar 2010, fundable score, funding should begin in October 2010  
Role: PI

Merit Review submission: Microsomal RNA role in regulatory pathway alterations of GWI. To be submitted Sept 1 2010

**Funded:**

**"The Use of Comprehensive Molecular Profiling with Network and Control Theory to Better Understand GWI and Mode Therapeutic Strategies"** Award Number W81XWH-09-2-0071,

DOD 6/2009 – 6/2011

Role: PI

R01AI065723 - 01 12/1/06 – 11/30/11

**Immunologic Mechanisms, Biomarkers and Subsets in CFS**

NIAID (PI MA Fletcher)

Goal of this project is to determine the immunologic basis for CFS pathogenesis

Role: Co-PI, 25% UM effort

R21AA016635-01 9/30/06-8/31/08

**R21: Neuropeptide Y and dipeptidyl-peptidase IV (CD26) in chronic fatigue syndrome**

NIAAA (PI MA Fletcher)

Goal of this

project to determine the relationship of neuropeptide Y and dipeptidyl-peptidase IV to natural killer cell cytotoxicity in CFS.

Role: Co-PI, 25% UM effort

Recent:

Merit Review 11/05 – 11/09

**Longitudinal follow up of GWI and CFS patients**

Veterans Administration

Study of immune function and clinical symptoms in patients with Gulf War Illness.

Role: PI

Merit Review 9/06 -8/09

**Gene Array Analysis of Gulf War Illness and Chronic Fatigue**

Veterans Administration

Gene Array Analysis of Gulf War Illness and Chronic Fatigue Syndrome

Role: PI

Research grant 03/07 – 03/09

**Mechanisms of Cytotoxic Cell Dysfunction in CFS**

CFIDS (PI N Klimas)

Study of killer cells in CFS

Role: PI, 15% UM effort

R01MH066697 09/04/03-6/30/08

**Psychobiological Processes and Health in HIV/AIDS**

NIMH R01 (PI G. Ironson)

This grant examines psychological and biological (CTL, NK, cortisol) predictors of disease progression in HIV/AIDS.

Role: Co-I, 5% UM effort

R01 9/30/06-8/31/10

**Virtual Cognitive Behavioral Therapy in Chronic Fatigue Syndrome**

NIMH (PI M Antoni)

Evaluate CBT utilizing phone and web based interventions

Role: Co-I, 5% UM effort

NIH sponsored Clinical Trial 2000 - 2008

**Immune Restoration with IL-2 in HIV infection – the ESPRIT study**

Role: Site PI

RO1 HL72712 09/30/02-08/31/07

HIV/HCV Co-Infection: HAART and CVD Pathophysiology

NIH/NHLBI (PI Hurwitz)

HAART medication has been implicated as a potential etiopathological source of the increased prevalence of cardiovascular disease risk in HIV infected persons. The study objective is to determine whether the data collected is described by the proposed pathophysiological model.

Role: Co-I

UO1- AI- 459940 8/1/00 to 7/31/04

NIAID (PI N Klimas)

Center for Multidisciplinary studies of CFS

This was a study of the modification of the stress response through a program of cognitive behavioral stress management and its effect on immune function in patients with chronic fatigue syndrome.

Role: PI

RO1 HL65668-05 09/27/01-07/31/05

NIH/NHLBI (PI Hurwitz)

RBC Mass, ANS Integrity & Syncope Susceptibility in CFS - 1 year no cost extension

The goal of the project was to study the pathogenesis of the chronic fatigue syndrome (CFS) which includes severe and debilitating fatigue, orthostatic intolerance, and the disruption of hematological, autonomic, and cardiovascular functions.

Role: Co-PI

Celgene Corp. 2003-2005

Clinical trial of Thalidomide

## Immune impact of Thalidomide in CFS

Role: PI

Dr Klimas has developed a set of outcome measures that can be utilized by multisite studies of CFS in clinical intervention or natural history trials. She is the chair of a consortium of clinicians interested in participating in clinical trials using this web-based format for assessment.

### 5d. Editorial Responsibilities:

Founding Editor: Journal of Chronic Fatigue Syndrome

Haworth Press (1992-2001)

Editorial Board, Journal of Chronic Fatigue Syndrome (2001 – current)

Ad hoc reviewer NEJM

Ad hoc Reviewer, Journal of Clinical Immunology

Ad hoc Reviewer, JAMA

Ad hoc Reviewer, Annals of Internal Medicine

Ad hoc Reviewer, AIDS

Ad hoc Reviewer, JAIDS

Ad hoc Reviewer, Psychosomatic Medicine

Ad hoc reviewer, Brain Behavior and Immunity

### 5e. Professional and Honorary Organizations:

International Association for CFS (previously AACFS), Current President

American Society for the Advancement of Science;

Association of Medical Laboratory Immunologists

Clinical Immunology Society

Association of Women in Science;

American Medical Woman's Association

University of Miami Medical Women

Miami Medical Women's Association (VP)

### 5f. Honors:

2004 – Honorary Degree, University of Catalonia, Barcelona Spain.

1998 - Fellow - Academy for Behavioral Medicine Research

1992 - Iron Arrow (University of Miami Honor Association)

1982 - Finalist Beecham Award, Southern Blood Club

1982 - Finalist Burroughs-Wellcome Young Investigator Award

1983 - Southern Medical Association Research Award

1984 - National Research Service Award

1985 - American Cancer Society Institutional Research Award

### 5g. Other Professional Activities:

Miami VAMC AIDS Clinical and Research Unit Developed and wrote the proposal for the Miami VAMC AIDS Clinical and Research Unit, which was one of 3 selected for funding. The proposal included 3 million dollars in construction funds as well as infrastructure support. This resulted in one of the top VA HIV/AIDS clinical and research programs in the US, which is still in operation. Dr Klimas is Director of AIDS Research and Co-director of the Clinical HIV/AIDS program at the Miami VAMC. 1987 to present

University of Miami and VAMC CFS and GWI Research Center : Initially funded with an NIH center grant, and since supported with NIH , VA, DOD, and private foundation grants, the center is a clinical , translational, and basic science center that integrates research across disciplines. Current studies include genomics, immune, neuroendocrine studies , a natural history study, and clinical trials. Dr. Klimas is Center PI and coordinates the research efforts of four research groups.

Canadian Government advisor in the development of the Clinical Case Definition for Chronic Fatigue Syndrome/Myalgic Encephalitis 2001, which is being revised in 2008.

Whittemore Peterson Institute - This University of Nevada institute is located in Reno, and is in its start up years, developing a clinical research program and a comprehensive clinic for patients with CFS/ME. Dr Klimas has been advising its executive committee on long term research goals. The institute is constructing a 25 million dollar facility which should open its doors in 2010.

6 month sabbatical CDC 2001 –, Molecular Epidemiology Program Viral Exanthems and Herpesvirus Branch, Developed the international protocol currently underway to define empirically CFS.

#### IACFS/ME

Dr. Klimas served as President of the International Association for Chronic Fatigue Syndrome (A national professional organization of investigators and clinicians) from 2005-2007 and was re-elected for another 3-year term in January of 2007. She organized the IACFS conference in Fort Lauderdale in January, 2007, and the conference in Reno Nevada in March 2009. Each of these conferences were attended by 400 patients and 350 professionals, and provided a unique opportunity for patients to meet and talk with leading international researchers and clinicians.

#### CFSAC: Chronic Fatigue Syndrome Advisory Committee

In 1996, Secretary for Health Donna Shalala chartered a special committee to advise the Department of Health and Human Services (DHHS) on policy regarding chronic fatigue syndrome (CFS), also known as chronic fatigue and immune dysfunction syndrome or CFIDS or Myalgic Encephalomyelopathy (ME). This committee, known as the DHHS Chronic Fatigue Syndrome Coordinating Committee (CFSAC), brought together officials representing various health agencies together with seven appointed members of the public to improve coordination of federal CFS programs. A year 2000 review of federal activities on CFS conducted by the General Accounting Office prompted several changes. Among them was the replacement of the CFSAC with a new committee, the CFS Advisory Committee (CFSAC), whose structure more closely matched other DHHS advisory bodies. Secretary Michael Leavitt most recently renewed the charter on August 30, 2006. Nancy Klimas served on this committee from 1997 to 2000. She was reappointed to another three-year term on the committee in 2007, which has been extended to 2011.

#### National Press Club in Washington, DC: The CDC's Chronic Fatigue Syndrome Public Education and Awareness Campaign.

Nancy Klimas was a participant at this event held on November 3, 2006. Present were Julie Gerberding, CDC, John Agwunobi, HHS, William Reeves, CDC and Anthony Komoroff, Harvard. Dozens of reporters from national and local media outlets across the United States were in attendance, and many others participated via phone link. Dr. Klimas remarked, —Historically, the lack of credibility afforded this illness has been a key obstacle to understanding it. Today, with solid evidence that CFS has identifiable biologic underpinnings, and with evidence that people with CFS experience a level of disability equal to that of patients with multiple sclerosis, advanced HIV disease and undergoing chemotherapy, I hope we can begin to put an end to the stigma surrounding this illness.” Dr. Klimas also focused on treatments, saying, —Although there's no single treatment—no hoped for ‘magic bullet’—that fixes the illness at its core, there are treatments that can improve symptoms, increase function and allow CFS patients to engage in activities of daily living. Current best practices for clinical care include a combination of symptom management, activity management and exercise therapies.”

Chronic Fatigue Centers for Research and Clinical Care: A newly conceived program hoping to use clinical care templates to help diagnose and manage complex CFS/ME cases while collecting research data and developing the patient base for clinical trials work. Dr. Klimas is the senior clinician developing the templates the first clinic to implement this format is the Chronic Fatigue Center in Kendall, FL which opened in 2010.

#### **5h. Consultantships**

1987 - 1990 - VA National AIDS Steering Committee

1987 - 1990 - VA National AIDS Research Subcommittee

1987 - 1991 - VA Train the Trainer National AIDS Education Program

1988 - Present - VA National AIDS Prevention and Counseling Training Program

1988 - Present - Special Review Committee, National VA AIDS Prevention and Education

1990 – 2000 - VA National HIV Therapeutics Advisory Committee

1991 - 2000 - Board of Directors, American Association for Chronic Fatigue Syndrome (An international professional organization of investigators and clinicians).

2002 – present - Board of Directors, American Association for Chronic Fatigue Syndrome (An international professional organization of investigators and clinicians).

1992 - Chairperson of the Program Committee for the First International Meeting: Chronic Fatigue Syndrome, held in Albany NY, sponsored by the AACFS, NIH and CDC.

1994 - Local Coordinator and Program Committee member for the Second International Meeting: Chronic Fatigue Syndrome research Conference, held in Ft Lauderdale, October 1994, sponsored by AACFS, NIH, CDC, and Univ. of Miami.

1994 - Chairperson of the Program Committee for the CFS Clinical Conference, held in Ft. Lauderdale, October 1994, sponsored by AACFS.

1991- 1997 Consultant to Center for Special Immunology, Inc., Ft. Lauderdale, FL.

1993 - 2000 - Board of Directors, American Association for Chronic Fatigue Syndrome.

1993- Present Medical Advisory Board, Chronic Fatigue and Immunodeficiency Syndrome Foundation.

1993- 1998 - Medical Advisory Board, Environmental Health Foundation.

2000 NIH State of the Science CFS Conference planning committee

2001 – present Name Change subcommittee, HHS CFS coordinating Committee

1999-present – CDC CFS Case Definition Revision Committee

2001 Canadian CFS Clinical Case definition expert panel

2001 CDC Expert Advisory Panel – long term outcomes study

2002 – present NIH reviewer and site visitor GCRC applications

2001- present Ad Hoc reviewer, Medical Research Council, United Kingdom.

2003 –present NIH reviewer CFS Special Emphasis Panel

2003 Brighton Collaboration on CFS Case Definition

2003 Elected to the Board of Directors, AACFS, 7 year term

2005 - 2009 President of the International Association for Chronic Fatigue Syndrome – this international organization of investigators and clinicians sponsors international and regional meetings, has developed a peer review journal, and works with government and regional groups to develop curricula and provider education programs.

2007 – 2011 CFSAC HHS Advisory committee to the Secretary of Health and Human Services

2010 – current Principal Proponent, National Gulf War Illness genomic bank and GWAS study, a VA cooperative study approved for planning, full protocol to be reviewed in Fall 2010.

5i. **TEACHING**

**Teaching Award:**

Woman Faculty Member of the Year, 1989, UM Medical Women.

**Current Teaching Responsibilities:**

Housestaff, Graduate Program, Medical School and Undergraduate lecturer (see lectures listed above)

General topics:

Clinical Immunology, Medical Laboratory Immunology, HIV Infection, Health and Human Values:

Psychoneuroimmunology, Allergy and Immunology, CFS, Gulf War Illness, Stress and Disease.

Internal Medicine, Ward Attending, VA Medical Center (3 months/ year)

Allergy Clinic rotations for medical students, housestaff and Harrington Latin American Scholars.

HIV and Immunology rotations for medical students, housestaff and Harrington Latin American Scholars

Nationally/Internationally helped to develop CME course work for clinicians in the diagnosis and treatment of CFS in collaboration with the CDC and CFIDS Association of America

**Dissertation Advising:**

Masters and Doctoral Students in Psychology (9 PhD candidates/3 MS candidates over last 5 years)

**6. SERVICE TO THE UNIVERSITY**

**6a. University Committees:**

1. Medical School Admissions Committee, 1984-1987.
2. VA Human Subjects IRB, 1987.
3. Faculty Sponsor, UM Medical Women, 1985 - Present.
4. UMHC Infection Control Committee, 1989 - 1992.
5. VAMC Research Space Committee, 1988 - 1989.
6. VA Research and Education Foundation Board of Directors – 1999- present
7. Search Committee - AIDS/HIV senior and junior research faculty
8. Selection Committee – applicants for AAMC Women in Medicine Leadership Program
9. Executive Committee, GCRC 2002 – present
10. Executive Committee, Behavioral Medicine Research Center, 1999 - present
11. University of Miami Committee on Rank Salary and Conditions of employment 2003-2004
12. VA Research Committee, alternate 2004-present
13. Faculty Senate Professional Conduct Panel 2003 – present
14. University of Miami Miller School of Medicine Self Study LCME Women and Minority subcommittee 2007- present

**6b. Clinical Responsibilities**

1. Co-director of VA Medical Center AIDS Clinical Unit,
2. HIV/AIDS Primary Care Clinic (Silver Team) – attending Monday clinic, and backing up Wednesday clinic. Daily oversight of 2 ARNP clinics.
2. General Medicine teaching attending, VAMC
3. Director, Miami VAMC Allergy Clinic, Tuesday MD clinic, and oversight of Wednesday RN clinic
4. Director of Allergy and Diagnostic Immunology outpatient clinic, University of Miami, Thursday Clinic
5. Director, CFS and GWI Clinical and Research Center, University of Miami and Miami VAMC

Consistently ranked —Outstanding” in the annual VAMC proficiency reporting, including clinical skills, teaching, productivity and administration.

**7. COMMUNITY ACTIVITIES**

Health Crisis Network, Co-Founder and Past Chair of the Medical Advisory Committee and Board of Directors. (Currently Co-Cure Foundation, a Miami Dade HIV related community health organization) 1984, 1985-89

Member, National Task Force on Women's Health Issues, National Organization for Women.(1995-present)

People with AIDS Coalition (PWAC), Board of Directors 1993-95

PWA Housing Coalition, Board of Directors 1993-95

Women's Emergency Network, Board of Directors 1998/99

Bessie Garrett Foundation – Homeless children outreach, Board of Directors 1999 – present

National Organization for Women, Women's Health Advisory Board, 1992-2002

PANDORA – advocacy for people with neuroinflammatory disorders, board of directors

## **APPENDIX G: Gordon Broderick, Ph.D. Curriculum Vitae**

### **CONTACT INFORMATION**

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Citizenship: Canadian Languages: French and English both spoken and written fluently

### **EDUCATION AND TRAINING**

Ph.D. Chemical Engineering	École Polytechnique de Montréal	1991-1994
Masters Chemical Engineering	McGill University	1988-1989
Bachelor Mechanical Engineering	McGill University	1980-1984

### **LICENSES.**

Ing. (P. Eng.)	Ordre des Ingénieurs du Québec	1986-present
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### **HONORS AND AWARDS**

Nightingale Award for Community Service, ME Society of Edmonton	2010
Associate Editor BMC Systems Biology	2010
Invited member US Veteran's Administration Committee on Gulf War Illness	2010
Teacher of the Year Award (Small Group Case Study)	2008
Dean of Graduate Studies and Research Award for best doctoral thesis.	1994
Natural Sciences and Engineering Research Council of Canada Award (Ph.D)	1991 - 1994
John S. Bates Centennial Fellowship (Ph.D)	1992 - 1993
R.M. Fowler Memorial Fellowship (Masters)	1988 - 1989
Natural Sciences and Engineering Research Council of Canada Award (Masters)	1988 - 1989
J.B. Lynch Foundation Award (Bachelor)	1980 - 1984

### **PROFESSIONAL /ACADEMIC POSITIONS HELD**

**Associate Professor – Dept. of Medicine, July 1, 2006 – Present**

**Faculty of Medicine and Dentistry, University of Alberta • Edmonton, AB**

Reporting to the Department Chair, establish independent research program in the area of computational medicine.

**Major Contributions:** (1) Principal investigator in computational research effort directed at understanding immune cell activation and population kinetics in chronic inflammatory illnesses (2) Actively developing agent-based model of the spatial dynamics of immune cell migration and signalling in the context of neuro-immune dysfunction. (3) Co-investigator /computational group leader in project directed at understanding gene regulatory mechanisms involved in injury and alloimmune response of kidney grafts.

**Project Leader – Cell Simulation 2002 – 2006**

**Institute for Biomolecular Design – University of Alberta • Edmonton, AB**

Reporting to the Executive Director, direct the research activities of an interdisciplinary group of 4-6 programmers and engineers working to create spatially discrete mathematical models of cell life. **Major Contributions:** (1) Lead architect in creation of scalable agent-based high-performance computational framework for whole cell simulation, (2) Developed scientific strategy behind IBM sponsorship of dedicated high-performance computing platform, (3) Developed first agent-based model of cell membrane chemistry / mechanics, (4) 2 U.S. Provisional Patents, 2 PCTs in 18 months.

**Consulting Biostatistician – Transplant Transcriptome Project****2004 – 2005****Alberta Transplant Applied Genomics Centre – University of Alberta • Edmonton, AB**

Reporting to the Executive Director, assist in the development of a strategy for ensuring the use of leading edge data analysis tools in supporting the research activities. **Major Contributions:** (1) Outlined standardised data handling and analysis pipeline, (2) Designed multivariate calibration filter for reconciliation of new micro-array architecture with previous generation, (3) Proposed strategy for establishing future biostatistics team, identifying key internal and external contributors.

**Principal Scientist****1999 – 2002****Noranda Technology Center • Pointe-Claire, QC**

Reporting to the Senior Vice-President Technology, formulate company-wide plan for developing strategic technologies in advanced process systems. Direct research efforts of project leaders in this area. Principal investigator with substantial discretion, define and execute high-impact research. **Major Contributions:** (1) Developed novel Monte Carlo signal processing technique for estimating particle size distributions from laser pulse patterns, (2) Lead research collaboration with McGill University directed at modelling complex spatial distributions using discrete stochastic automata, (3) Lead high-risk collaboration with University of Lund aimed at innovative applications of artificial reasoning (reinforcement learning) for advanced diagnostics.

**Visiting Scientist****2000 – 2001****McGill School of Computer Science / Montreal General Hospital • Montreal, QC.**

Responsible for computational aspects of multidisciplinary effort directed at elucidating pathway kinetics and specificity of steroid-containing drugs in treating immune disorders using gene expression time course sequences. **Major Contributions:** (1) Completed n-way projection analysis of multi-tissue time-course responses to steroid impulse stimuli, (2) Implemented and evaluated algorithm for multivariate linear filtering, discovery and class prediction for AML and ALL-type leukemia, (3) Conducted comparison of results from PCA and self-organising maps (SOM) for extraction of features from gene expression in yeast cell cycle data.

**Senior Scientist****1995 – 1999****Noranda Technology Center • Pointe-Claire, QC**

Initiate and actively lead new research projects using discretionary funding to identify promising new technologies, develop proof of concept. Effectively coordinate academic and private consultants. Appointed by Senior Vice-President Technology to committee reviewing Noranda's high-impact research initiatives. **Major Contributions:** (1) Patented innovative energy-saving refining strategy. (2) Established partnership with Colorado State University, and Hong Kong University in evolutionary programming. (3) Established research partnership with University of Waterloo for wavelet-based adaptive estimation and control. (4) Defined, and obtained funding for a 2-year collaborative research with McGill University on cellular automata (CA).

**Research Engineer/Project Leader****1989 - 1995****Noranda Technology Center • Pointe-Claire, QC**

Optimize process performance by applying mathematical modeling and simulation techniques. Develop and maintain close ties with academic institutions and other private research facilities to help identify promising new technologies, transfer, and adapt these to meet strategic needs. Act as a technical leader in statistical process modeling. **Major Contributions:** (1) Developed detailed product quality models based on intrinsic material properties. (2) Constructed novel multivariate statistical approach to quality monitoring. (3) Successfully implemented PLS model-based system for on-line prediction and quality optimization.

**RESEARCH****Invited presentations.**

Broderick, G. 2010. Networked Regulatory Systems in Complex Illness. Developmental Center for AIDS Research. University of Miami, Miami, FL, February 16.

Broderick, G. 2010. A Systems Biology Approach to Understanding Homeostasis Reset - the CFS/GWI Experience. John P. Hussman Institute for Human Genomics, University of Miami, Miami, FL, February 16.

Broderick G. 2009. Subtle Immune Signatures in Chronic Fatigue Syndrome and Gulf War Illness. From Infection to Neurometabolism: A Nexus for CFS. The Banbury Centre, Cold Spring Harbor Laboratory, Long Island, NY, Sept. 13-16, Closed session.

Broderick G, Klimas N, 2009. Immune Network Dysfunction in Gulf War Illness. Research Advisory Committee, U.S. Department of Veterans Affairs, Boston, MA, June 29-30.

Broderick G. 2009. Discovery Learning: A Preceptor's Impressions. Academic Half-day, Geriatric Division, Glenrose Rehabilitation Hospital, Edmonton, AB, June 16.

Broderick G, 2009. CFS/ME Lifting the Veil on a Complex Illness. Guest Speaker, M.E. Awareness Day, M.E. Society of Edmonton, Edmonton, AB, May 11.

Broderick G, 2009, Chronic Fatiguing Illnesses: Immune Signatures and Beyond. Research Roundtable Guest Speaker. The CFIDS Association of America, Chicago, IL, May 3.

Broderick G, Fletcher MA, Vernon SD, Klimas N, 2009, Isolating Characteristic Immune Signals under Challenge in Gulf War Illness. Int Assoc CFS/ME, Reno, NV, March 12-15.

Klimas N, Fletcher MA, Broderick G, 2008, Gulf War Syndrome: A Systems Biology Approach. Keynote address, Acad. Behavioral Medicine Ann. Meeting, Lake Louise, AB, June 14-16.

Vernon SD, Fuite J, Broderick G. 2008. Genetic Variation and Altered Immune Activity in Chronic Fatigue Syndrome. 6<sup>th</sup> Int Conf HHV-6 Foundation, Int Symp on Viruses in Chronic Fatigue Syndrome and Post-viral fatigue, Baltimore, MD, June 22-23.

Broderick G, Schaefer CF, Einecke G, Halloran PF, 2008, Identifying Changes in Gene Regulatory Motifs in Rejection of Human Kidney Transplants, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, Jan. 25.

Vernon SD, Fuite J, Broderick G. 2008. Neuroendocrine and Immune Network Re-modeling in Chronic Fatigue Syndrome. Integrative Neural Immune Interest Group (INIIG) Lecture Series, National Institute of Mental Health, Bethesda, MD, March 4.

Broderick G. 2008. Agent-based Models in Medicine: the Promise and the Challenges. Invited keynote speaker. SwarmFest 2008, Northwestern University Feinberg School of Medicine, Chicago IL, May 12-13.

Broderick G, Ben-Zvi A, Aslakson E, Klimas N, Vernon SD. 2007. An MPC-guided Approach for the Regulation of Cortisol in a Hypothalamic-pituitary-adrenal Axis Model. Invited talk, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, Aug. 29.

Broderick G, Bolshin C, Aspler AL. 2007. Evidence of Lymphocyte Imbalance in Wichita Study of CFS sufferers. Centers for Disease Control and Prevention (CDC), Atlanta, GA, August 27.

Broderick, G, 2007. Delayering the Data: Revealing Rejection in Human Kidney Transplants by isolating Gene Set Co-expression. Invited talk, Using the "Omic" technologies to Phenotype Disease: A Satellite Symposium of the 9th Banff Conference on Allograft Pathology. Edmonton, AB, June 18-19.

Broderick G, 2006. Composite Features of Fatigue. Invited Speaker, Computational Challenge (Team 2), Centers for Disease Control and Prevention (CDC), Atlanta, GA, March 15.

Broderick G, 2006, The Virtual Cytoplasm: Exploring Complex Intra-cellular Kinetics. Department of Mathematical & Statistical Sciences, University of Alberta, Edmonton, Alberta, Canada, Feb. 27.

Broderick G, 2005. CFS: From Constructs to Mechanisms", Invited Speaker, CDC Computational Challenge (Team 2), Banbury Centre Workshop, From Markers to Models: Integrating Data to Make Sense of Biologic Systems, Cold Spring Harbor Laboratory, Long Island, N.Y., Sept. 18-21, Closed session.

Broderick G, 2005. The Virtual Cell Model: Exploring Emergent Behaviour in Nature's Inherent Integration Milieu, Invited Speaker, Realistic Modelling of Biological Systems: A First International Workshop, Weizmann Inst. of Science, Rehovot, Israel, May 2-4.

Broderick G, 2005. Integrating a Model Biological Membrane and Virtual Cytoplasm. Invited Speaker, Realistic Modelling of Biological Systems: A First International Workshop, Weizmann Inst. of Science, Rehovot, Israel, May 2-4.

Broderick G, 2005. Building a Large-scale Functional Model of a Biological Membrane. Invited Speaker, Faculty Talk, Center for Complexity Science, Weizmann Inst. of Science, Rehovot, Israel, May 1.

Broderick G, 2004. A Parallel Particle-based Approach to Whole-cell Modelling. Invited Speaker, Banbury Centre Conference on Integrating Disparate Data to Simulate Lymphocyte Function, Cold Spring Harbor Laboratory, Long Island, N.Y., Sept. 19-22, Closed session.

Broderick G, Ru'aini M, Winters P, Chan E, Ellison MJ, 2004. Towards a Life-like Virtual Cell Using Discrete Automata. Departmental Seminars in Chemical and Materials Engineering, University of Alberta, Edmonton, Alberta, Canada, December 2.

### **Grant support.**

#### Awarded

##### **U.S. Department of Defense**

**July 2009 – June 2011**

The Use of Comprehensive Molecular Profiling with Network and Control Theory to Better Understand GWI and Model Therapeutic Strategies  
Budget (Co-investigator U of A)

##### **CFIDS Association of America**

**March 2009 (1.5 year term)**

Molecular Patterns of Persistent Immune Activation in a Post-infectious Adolescent Cohort  
Principal Investigator,  
Budget (PI)

##### **Alberta Heritage Foundation for Medical Research**

**May 2009 – April 2014**

Title of team program: Etiology of Inflammatory Bowel Disease: Gene, Microbe, and Environment Interactions  
Budget (Co-investigator U of A)

##### **University of Alberta, Faculty of Medicine and Dentistry**

**July 2009 - August 2009**

Henry Anton Deutsch Medical Summer Research Award (in support of summer student research)

##### **University of Alberta, Faculty of Medicine and Dentistry**

**July 2009 - August 2013**

Title of Program: Biomarkers for Gulf War Illness (Research Award in Support of Summer Studentships in Broderick Laboratory)

#### **NIH**

A Prospective Study of CFS in Adolescents

**January 2008 – January 2010**

Co-investigator; Principal Investigator: Dr. R. Taylor, University of Illinois in Chicago

Budget (Co-investigator U of A)

#### Pending

##### **National Institutes of Health (NIH)**

**July. 2010 – June. 2014**

Information Thermodynamics of Immune Networks in Chronic Inflammation, Fatigue and Cancer. R01 Eureka Program.  
Budget (PI)

##### **U.S. Department of Defense**

**July 2010 – June 2012**

Autoantibody profiling to identify predisposing auto-reactivity to myalgia-arthralgia-fatigue syndromes.

Budget (Co-investigator U of A)

**U.S. Department of Defense**

**July 2010 – June 2013**

Theory-driven Models for Correcting “Fight or Flight” Imbalance in Gulf War Illness.

Budget (PI)

**National Institutes of Health (NIH)**

**Dec. 2009 – Nov. 2014**

Study of Chronic Fatigue Syndrome using comprehensive molecular profiling with network and control theory. R01.

Budget (Co-investigator U of A)

*Administrative supplement:* Study of Chronic Fatigue Syndrome using comprehensive molecular profiling with network and control theory.

Budget (Co-investigator U of A)

**U.S. Department of Veterans Affairs**

**Oct. 2009 – Sept. 2012**

Placebo controlled phase II double blind randomized clinical trial of inosine pranobex in GWI and CFS.

Budget (Co-investigator U of A)

Previously held

**University of Alberta, Faculty of Medicine and Dentistry      July 1, 2006 - June 30, 2009**

Start-up Grant N031000099: Mechanistic analysis of microarray signatures in transplant rejection.

**Genome Canada**

**July 2006 – December 2007**

Transplant Transcriptome Project

Co-investigator; Principal Investigator: Dr. P.F. Halloran, University of Alberta

Budget (Co-investigator U of A)

**NSERC**

Real-time adaptive pattern recognition using cellular automata      **July 1997 – July 1999**

Industry co-investigator; Principal Investigator: Dr. D. Thérien, McGill University

Collaborative Research and Development (CRD)

Optimisation of high-yield sulphite pulping

**May 1990 – April 1994**

Industry co-investigator; Principal Investigator: Dr. J.L. Valade, Dr. J. Paris, École Polytechnique de Montréal

Collaborative Research and Development (CRD)

**Reviewing activities.**

Review of candidates for faculty positions.

2008. Candidate Senior Lecturer; Faculty of Life Sciences; University of Bar-Ilan, Israel

2006. Candidate Senior Lecturer; Dept. of Bio-medical Eng., Ben Gurion University, Israel

Grant reviews

2010 Health Research Awards, the Health Research Board (HRB) of Ireland, Dublin, Ireland

Chief Scientist Office of the Scottish Government Health Directorate, Edinburgh, UK

2009 U.S.-Israel Bi-national Science Foundation, Jerusalem, Israel

Chief Scientist Office of the Scottish Government Health Directorate, Edinburgh, UK (2 grant proposals)

Microsoft Research PhD Scholarship Programme. Microsoft Research, Cambridge, UK (2 proposals)

Canadian Institutes of Health Research (CIHR)

Natural Sciences and Engineering Research Council of Canada (NSERC)

2006. The Centre for Complexity Science, Jerusalem, Israel

Peer review of publications.

2010. Associate Editor, BMC Systems Biology, BioMed Central, London, UK (Impact 3.71)

2009. Journal of Immunology  
 Journal of Infectious Diseases  
 BMC Medical Genomics  
 American Journal of Transplantation  
 Cellular and Molecular Life Sciences  
 Brain, Behavior, and Immunity (2 manuscripts)

2008. Molecular Medicine  
 The Journal of Physical Chemistry  
 American Journal of Transplantation (2 manuscripts)  
 PLoS One

2007. Transplantation (with Dr. Bruce Kaplan, University of Illinois in Chicago).  
 Bioinformatics; Oxford Press (2 manuscripts).  
 American Journal of Transplantation (2 manuscripts)  
 BMC Neurology  
 Theoretical Biology and Medical Modeling

2006. PLoS Computational Biology

2002 and previous  
 Canadian Journal of Chemical Engineering (regularly requested)

Peer review of conference abstracts

2009 Department of Medicine Research Day Graduate Student Poster Session (6 posters)

**Bibliography.**

Refereed journals.

Fletcher MA, Rosenthal M, Antoni M, Ironson G, Zeng X-R, Harvey J, Barnes Z, Hurwitz B, Levis S, Broderick G, Klimas NG. Neuropeptide Y: a marker for stress and symptom severity in chronic fatigue syndrome. In preparation.

Broderick G, Andrea Kreitz, Fuite J, Fletcher MA, Vernon SD, Klimas NG. Remodeling of Lymphocyte-cytokine Networks in Gulf War Illness under Challenge. *Brain Behav Imm.* 2010; Under review.

Broderick G, Fletcher MA, Gallagher M, Vernon SD, Klimas NG. Characteristic Immune Response to Exercise Challenge in Gulf War Illness. *BMC Clin Pathol.* 2009; Under review.

Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. Circadian rhythms in cytokine secretion in chronic fatigue syndrome. *Brain Behav Immun.* 2010 Jun 19.

Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. Formal Analysis of Cytokine Networks in Chronic Fatigue Syndrome. *Brain Behav Immun.* May 4, 2010. [Epub ahead of print].

Fletcher MA, Zeng XR, Maher K, Levis S, Barry H, Antoni M, Broderick G, Klimas NG. Biomarkers in chronic fatigue syndrome: Evaluation of natural killer cell function and dipeptidyl peptidase IV. *PLoS ONE.* 25 May, 2010 | 10.1371/journal.pone.0010817.

Nakamura T, Schwander SK, Donnelly R, Ortega F, Togo F, Broderick G, Yamamoto Y, Cherniack NS, Rapoport D, Natelson BH. Cytokines across the night in chronic fatigue syndrome with and without Fibromyalgia. *Clin Vaccine Immunol.* 2010 Feb 24. [Epub ahead of print]

Ben-Zvi A, Vernon SD, Broderick G. 2008. Model-based Therapeutic Correction of Hypothalamic Pituitary Adrenal Axis Dysfunction. *PLoS Comput Biol* 5(1): e1000273. doi:10.1371/journal.pcbi.1000273.

Aspler AL, Bolshin C, Vernon SD, Broderick G. 2008. Evidence of Inflammatory Immune Signaling in Chronic Fatigue Syndrome: A Pilot Study of Gene Expression in Peripheral Blood. *Behav Brain Funct* Sep 26;4(1):44. doi:10.1186/1744-9081-4-44.

Fuite J, Vernon SD, Broderick G. 2008. Neuroendocrine and Immune Network Re-modeling in Chronic Fatigue Syndrome: An Exploratory Analysis. invited submission, *Genomics* 92(6): 393-399. doi:10.1016/j.ygeno.2008.08.008 (issue cover).

Zhao J, Ridgway D, Broderick G, Kovalenko A, Ellison M, 2008. Extraction of elementary rate constants from global network analysis of *E. coli* central metabolism. *BMC Systems Biology* 2:41 doi:10.1186/1752-0509-2-41.

Ridgway D, Broderick G, Ru'aini M, Winter P, Lopez-Campistrous A, Ellison MJ. 2008. Coarse-grained molecular simulation of diffusion and reaction kinetics in a crowded virtual cytoplasm. *Biophys J* May 15;94(10):3748-59.

Mueller TF, Einecke G, Reeve J, Sis B, Mengel M, Jhangri G, Bunnag S, Cruz, J, Wishart D, Meng C, Broderick G, Kaplan B, Halloran PF, 2007. Microarray analysis of rejection in human kidney transplants using pathogenesis-based transcripts sets. *Am J Transplant* 7(12): 2712-2722.

Famulski KS, Broderick G, Einecke G, Hayl K, Cruz J, Sis B, Mengel M Halloran PF. 2007. Transcriptome analysis reveals heterogeneity in the injury response of kidney transplants. *Am J Transplant* 7: 1-13.

Einecke G, Broderick G, Sis B, Halloran PF, 2007. Early loss of renal transcripts in kidney allografts: relationship to morphologic changes and alloimmune effector mechanisms. *Am J Transplant*, 7(5): 1121-1130.

Broderick G, Rubin E, 2006. The Realistic Modelling of Biological Systems: A Workshop Synopsis. *ComPlexUs* 3:217-230.

Ridgway D, Broderick G, Ellison MJ, 2006. Accommodating space, time and randomness in network simulation. Invited paper. *Curr Opin Biotechnol* 17:1-6.

Broderick G, Craddock RC, Whistler T, Taylor R, Klimas N, Unger ER, 2006. Identifying illness parameters in fatigue syndromes using classical projection methods. *Pharmacogenomics* 7 (3): 407-419.

Whistler T, Craddock RC, Taylor R, Broderick G, Klimas N, Unger ER, 2006. Gene expression correlates of fatigue. *Pharmacogenomics* 7(3): 395-405.

Craddock RC, Taylor R, Broderick G, Whistler T, Klimas N, Unger ER, 2006. Exploration of statistical dependence between illness parameters using the Entropy Correlation Coefficient. *Pharmacogenomics* 7(3): 421-428.

Lopez-Campistrous A, Semchuk P, Burke L, Palmer-Stone T, Brokx SJ, Broderick G, Bottorff D, Weiner JH, Ellison MJ, 2005. Localization, Annotation & Comparison of the *Escherichia coli* K-12 Proteome under Two States of Growth, *Mol. Cell. Proteomics*, May 19; 10.1074/mcp.D500006-MCP200.

Broderick G, Ru'aini M, Chan E, Ellison MJ, 2004. A Life-like Virtual Cell Membrane Using Discrete Automata. Invited Paper, *In Silico Biology* 5: 0016.

Fenton TE, Budman HM, Pritzker MD, Bernard E, Broderick G, 2003. Modeling and Simulation of Oriented Strandboard Pressing. *Ind. Eng. Chem. Res.* 42(21): 5229 – 5238.

Tessier P, Broderick G, Plouffe P, 2002. Competitive Analysis of North-American Newsprint Producers Using Composite Statistical Indicators of Product and Process Performance. *Pulp & Paper Canada* 103(5): T140-143.

Knapp T, Budman H, Broderick G, 2001. Adaptive Control of a CSTR with a Neural Network Model. *Journal of Process Control*, 11: 53-68.

Tessier P, Broderick G, Plouffe P, 2001. Competitive Analysis of North-American Newsprint Producers Using Composite Statistical Indicators of Product and Process Performance. *Tappi Journal* 84(3): 81.

Tessier P, Broderick G, Desrochers C, 2000. Chip Size Distribution for an Ultra-high-yield Sulfite Process. *Tappi Journal* 83(4): 76.

Broderick G, Handle B, Paschen P, 1999. Strategies for Optimal Operation of the Tellurium Electrowinning Process. *Metallurgical and Materials Transactions B*, 30B(1): 5-13.

Broderick G, Cacchione E, Heroux Y, 1998. The Importance of Distribution Statistics in the Characterisation of Chip Quality. *Tappi Journal*, 81(2): 131-142.

Tessier P, Broderick G, 1997. Industrial Implementation of Motor Load and Freeness Control of Chemimechanical Pulp Refiners. *Tappi Journal*, 80(12): 135-142.

Handle B, Broderick G, Paschen P, 1997. A Statistical Response Surface Study of the Tellurium Electrowinning Process. *Hydrometallurgy*, (46): 105-120.

Broderick G, Lanouette R, Valade JL, 1997. Optimizing Refiner Operation with Statistical Modelling. *Canadian Journal of Chemical Engineering*, 75(1): 79-87.

Broderick G, Paris J, Valade JL, 1996. Fibre Development in Chemimechanical Pulp Refining. *Tappi Journal*, 79(4): 193-201.

Broderick G, Paris J, Valade JL, 1996. The Impact of High-yield Pulping Pretreatment Conditions on Spent Liquor Toxicity and Oxygen Demand. *Paperi ja Puu*, 78(1-2): 43-50.

Broderick G, Paris J, Valade JL, Wood J, 1996. Linking the Fibre Characteristics and Handsheet Properties of a High-Yield Pulp. *Tappi Journal*, 79(1): 161- 169.

Broderick G, Paris J, Valade JL, 1995. Factors Affecting the Optimal Performance of a High-Yield Pulping Operation. *Canadian Journal of Chemical Engineering*, 73(3): 391-399.

Broderick G, Paris J, Valade JL, Wood J, 1995. Applying Latent Vector Analysis to Pulp Characterization. *Paperi ja Puu* 77(6-7): 410-418.

Broderick G, Paris, J, Valade JL, 1995. A Composite Representation of Pulp Quality. *Chemometrics and Intelligent Laboratory Systems* 29(1): 19-28.

Broderick G, Valade JL, Paris J, 1993. High Yield Sulphite Pulping Based on a Plackett-Burman Design. *Pulp and Paper Canada* 94(9): T248-251.

**Abstracts**

Katz B, Fletcher MA, Taylor R, Vernon SD, Broderick G. 2010. Cytokine Expression as a Potential Prognostic Indicator in Post-infectious Fatigue. Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR), Chicago, IL, Oct. 3-7.

Yang C, Vernon SD, Broderick G. 2009. Cognitive Performance in a Population-based Cohort of CFS Patients. Poster presentation. 95th Annual Clinical Congress, American College of Surgeons, Chicago, IL, Oct. 11-15.

Yang C, Vernon SD, Broderick G. 2009. Cognitive Performance in a Population-based Cohort of CFS Patients. Poster presentation. 42nd Annual Summer Students Research Day, Faculty of Medicine and Dentistry, University of Alberta, Oct. 17: poster 154.

Broderick G, Fletcher MA, Vernon SD, Klimas N, 2009, Isolating Characteristic Immune Signals under Challenge in Gulf War Illness. Int Assoc CFS/ME, Reno, NV, March 12-15: Oral session Abstracts/ Latest Research in Immunology, abstract #3.

Broderick G, Fuite J, Fletcher MA, Vernon SD, Klimas N, 2009, Remodeling of Lymphocyte-cytokine networks in Gulf War Illness under Challenge. Int Assoc CFS/ME, Reno, NV, March 12-15: Poster Abstracts/ Latest Research in Immunology, poster abstract #1.

Fuite J, Vernon SD, Broderick G. 2008, Re-modelling of neuroendocrine-immune interaction in Chronic Fatigue Syndrome. Department of Medicine Research Day, University of Alberta, Edmonton AB, May 29: Abstract 67.

Fuite J, Vernon SD, Broderick G. 2008. Understanding chronic fatigue using comparative cross-scale analysis of information networks. Systems Biology: Global Regulation of Gene Expression, Cold Spring Harbor Laboratory, Long Island, NY, March 27 - March 30. Abstract 47.

Fuite J, Vernon SD, Broderick G, 2007, Neuro-endocrine and Immune Network Re-modeling in Chronic Fatigue Syndrome: An Exploratory Analysis. 7th Int. Conf. for the Critical Assessment of Microarray Data Analysis (CAMDA 2007), December 13-14, Valencia, Spain: Abstract 32.

Broderick G, Einecke G, Famulski KS, Halloran PF, 2007. Dysregulation of Epithelial Repair Dynamics in Rejecting Mouse Kidney Transplants. American Society of Nephrology, Renal Week 2007, October 31- November 5, San Francisco, CA (Poster).

Broderick G, Einecke G, Mueller TF, Sis B, Halloran PF, 2007. Delaying the Data: Revealing Rejection in Human Kidney Transplants by Isolating Gene Set Co-Expression Patterns. American Transplant Congress, San Francisco, USA, May 5-9, Abstract 484: 273-274.

Einecke G, Broderick G, Sis B, Halloran PF, 2007. Early Loss of Renal Transcripts in Kidney Allografts: Epithelial Response to Injury and the Relationship to Morphologic Changes. American Transplant Congress, San Francisco, USA, May 5-9: Abstract 683: 325.

Broderick G, Schaefer C, Einecke G, Halloran PF, 2007. Identifying Changes in Gene Regulatory Motifs in Rejection of Human Kidney Transplants. Systems Biology: Global Regulation of Gene Expression, Cold Spring Harbor Laboratory, Long Island, N.Y., March 29 - April 1. Abstract 35.

Douglas DN, Lewis J, Broderick G, Bond D, Kneteman NM, 2006. Transcriptional profiling of HCV infection and treatment with interferon alpha in the chimeric mouse model for HCV infection. Proc. 3rd Int Dominique Dormont Conference (2006): Viral Escape to Therapy in Chronic Infections, Ancienne Faculté de Medicine et de Pharmacie - Place de la Victoire, Bordeaux, France, Dec.7-9, Abstract O15 : 38.

Einecke G, Reeve J, Sis B, Broderick G, Halloran PF, 2006. The Alloimmune Response Induces Allospecific Changes of the Transcriptome within 24 Hours in Mouse Kidney Transplants. American Society of Nephrology, Renal Week 2006, Nov. 14-19, San Diego, CA. (Poster)

Broderick G, 2005. The Virtual Cell Model: Exploring Emergent Behaviour in Nature's Inherent Integration Milieu. Invited Speaker, Realistic Modelling of Biological Systems: A First International Workshop, Weizmann Inst. of Science, Rehovot, Israel, May 2-4.

Broderick G, 2005. Building a Large-scale Functional Model of a Biological Membrane. Invited Speaker, Faculty Talk, Weizmann Inst. of Science, Rehovot, Israel, May 1.

Broderick G, Ru'aini, Ellison MJ, 2004. A Parallel Particle-based Approach to Whole-cell Modelling. Int. E.coli. Alliance (IECA) Conference on Systems Biology, Program and Abstracts, Banff, Alberta, Canada, June 18-22: 13.

Lopez-Campistrous A, Semchuk P, Burke L, Palmer-Stone T, Garen G, Brokx S, Broderick G, Bottorff D, Locke T, Weiner J, 2004. Localisation and Annotation of the Escherichia coli K-12 Proteome in the Presence and Absence of Amino Acids. Int. E.coli. Alliance (IECA) Conference on Systems Biology, Program and Abstracts, Banff, Alberta, Canada, June 18-22.

Broderick G, Ru'aini M, Winters P, Chan E, Ellison MJ, 2004. Towards a Life-like Virtual Cell Using Discrete Automata. Departmental Seminars in Chemical and Materials Engineering, University of Alberta, Edmonton, Alberta, Canada, December 2.

Ellison MJ, Broderick, G, Ru'aini M, Bottorff D, Weiner J, 2003. A massively Parallel Arithmetic Approach to Whole-cell Modelling in 4-D. First Int. E.coli. Alliance (IECA) Conference on Systems Biology, Program and Abstracts, Tsuroka, Japan, June 23-25: 24.

Tessier P, Broderick G, 2000. Example Applications of Multivariate Statistical Analysis in the Pulp and Paper Industry. Proc. PAPTAC Conf., Montreal, January 31-Feb 4.

Graff S, Broderick G, Tessier P, Leger R, 2000. Predictive Models of Effluent Treatment at Fraser Paper in Edmundston. Proc. Tappi 2000 Environmental Conf., Denver, CO, May 7-10.

Pudlas M, Broderick G, Tessier P, 2000. Assessing Competitive Position Using Multivariate Statistical Analysis of Pulp Quality. Proc. PACWEST Conference 2000, Pulp and Paper Technical Assoc. of Canada, Jasper, A.B., June 1-4.

Tessier P, Broderick G, Desrochers C, Bussiere S, 1997. Motor Load and Freeness Control of Chemimechanical Pulp Refining: Industrial Results. European Control Conference, Brussels, Belgium, July 1-4.

Broderick G, Tessier P, 1996. On-line Fibre Quality Sensors: What They Should Measure and Why. Pulp Expert User Seminar, Pulp Expert Oy, Montreal, September 30.

Broderick G, 1996. PLS and Latent Vector Modelling: A Decision Support Tool. Technical Session, CPPA Process Control Committee, Vancouver, September 23.

Broderick G, Paris J, Valade JL, 1993. The Impact of Processing Conditions on Spent Sulphite Liquor Toxicity. Proc. 43rd Canadian Chemical Engineering Conf., Ottawa, Ont., Oct. 3-6: 206.

Broderick G, Valade JL, Paris J, 1991. Identifying Dominant Process Factors in High Yield Sulphite Pulping Using a Plackett-Burman Design. Proc. 41st Canadian Chemical Engineering Conf., Vancouver, Oct. 6-9: 19-5.

#### Proceedings

Benz-Zvi A, Lee JM, Broderick G, 2009, An MPC-based Approach to Robust Control of a Hypothalamic-Pituitary-Adrenal Axis Model. 6<sup>th</sup> IFAC Symposium on Robust Control Design, Haifa, Israel, June 16-18: paper TuM1.1.

Tessier P, Broderick G, Plouffe P, 2000. Competitive Analysis of North-American Newsprint Producers Using Composite Statistical Indicators of Product and Process Performance. Proc. Control Systems 2000, Pulp and Paper Technical Assoc. of Canada, Victoria, BC, May 1-4: 263-266.

Broderick G, Tessier P, Desrochers C, Bussiere S, 1998. On-line Prediction of Newsprint Furnish Quality Using Multivariate Statistical Models. Proc. Control Systems 98, Finnish Society of Automation, Porvoo, Finland, Sept. 1-3: 135-142.

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Bolduc JS, Broderick G, Therien D, 1997. From Stability to Tracking: Robustness of Cellular Automata Based Controllers. Proc. IEEE 1st International Conference on Intelligent Processing Systems", Beijing, China, October 28-31, Vol. 1: 619-624.

Broderick G, Cacchione E, Heroux Y, 1996. The Importance of Distribution Statistics in the Characterisation of Chip Quality. Proc. Tappi Pulping Conf., Nashville, TN, Oct. 27-31, Book 2: 535-549.

Broderick G, Paris J, Valade JL, 1994. Fibre Development in Chemimechanical Pulp Refining. Proc. Tappi Pulping Conf., San Diego, CA, Nov. 6-10: 435-443.

Broderick G, Paris J, Valade JL, 1994. Monitoring Composite Pulp Quality. Proc. 80th Annual Meeting CPPA Technical Section, Montreal, Feb. 1-4: B35-B40.

Lanouette R, Broderick G, Valade JL, 1994. L'Optimisation du raffinage par la modélisation statistique. Proc. Conférence Technologique Estivale, Pointe-au-Pic, June 1-3 : 1-9.

Broderick G, Valade JL, Paris J, 1992. High Yield Sulphite Pulping Based on a Plackett-Burman Design. The 78th Annual Meeting CPPA Technical Section, Montreal, Jan. 28-31: A141-A146.

Patents.

Broderick G, Ru'aini M, Ellison MJ, 2004. System and Method for Simulating Biological Cell Membrane Processes. Patent Cooperation Treaty PCT/CA2005/000795 (May 25, 2005), U.S. provisional patent appl. #60/575,089 (May 28, 2004).

Ellison MJ, Broderick G, Ru'aini M, Bottorff D, Wishart D, 2004. System and Method for Simulating Living Cell Processes. Patent Cooperation Treaty PCT/CA2004/000369 (May 5, 2004), U.S. provisional patent appl. #60/453,257 (March 10, 2003).

Broderick G, Lanouette R, Valade JL, 1995. Optimal Energy Refining Method for the Mechanical Treatment of Wood Fibres. Canadian patent 2,150,647 (November 30, 95), U.S. patent 5,540,392 (July 30, 1996).

**TEACHING**

**Undergraduate Teaching Activities.**

**DMED 514** – Cardiovascular

Winter 2010

Small group facilitator

Overall effectiveness rating

9.8/10.0

**MED524** Neurosciences and Organs of Special Senses (A)

Small group facilitator

Overall effectiveness rating

9.4/10.0

**DMED 512** - Infection, Immunity and Inflammation

Fall 2009

Small group facilitator

Overall effectiveness rating

9.4/10.0

**MED522** Reproductive Medicine and Urology

Small group facilitator

Overall effectiveness rating

10.0/10.0

**MED526/DDS520** Patient-centred Care

Small group facilitator

Overall effectiveness rating

TBD

**MED524/DDS507** Neurosciences and Organs of Special Senses

Winter 2009

Discovery learning (DL) small group facilitator

Part A. Overall effectiveness rating

10.0/10.0

Part B. Overall effectiveness rating

TBD

**MED515** Community Health

Small group facilitator

Overall effectiveness rating

9.8/10.0

**MED526/DDS520** Patient-centred Care II

Small group facilitator

Section III. Session on Fibromyalgia and Allied Conditions

Section IV. Session on Mental Health Site Visits

Section IV. Session on Alzheimer's

Overall effectiveness rating

8.0/10.0

**MED516 /DDS510** Patient-centred Care I  
Small group facilitator; Session on Voluntariness

Fall 2008

**MED526 /DDS520** Patient-centred Care I  
Small group facilitator; Session on diabetes

**MED521 /DDS506** – Gastroenterology and Nutrition  
Discovery learning (DL) small group facilitator  
Overall effectiveness rating 9.8/10.0

Substitute Teaching as Discovery Learning (DL) small group facilitator in:  
**DMED 512** - Infection, Immunity and Inflammation  
**DMED 513** – Endocrinology and Metabolism

**DMED 514** – Cardiovascular, Pulmonary and Renal Systems Winter 2008  
Discovery learning (DL) small group facilitator  
Renal Block; Overall effectiveness rating 9.4/10.0  
Pulmonary Block; Overall effectiveness rating 9.1/10.0  
Cardiology Block; Overall effectiveness rating 9.4/10.0.

**DMED 512** - Infection, Immunity and Inflammation Fall 2007  
Discovery learning (DL) small group facilitator  
Overall effectiveness rating 9.2/10.0

**DMED 514** - Cardiovascular, Pulmonary and Renal Systems. Winter 2007  
Discovery learning (DL) small group facilitator;  
Renal Block; Overall effectiveness rating 9.4/10.0  
Pulmonary Block; Overall effectiveness rating 10.0/10.0.

### **Undergraduate Research Supervision / Co-supervision**

Mr. Scott deGraff Summer 2010  
Immune Network Dynamics in Gulf War Illness

Mr. Landon Berger Summer 2009  
Immune Biomarkers of Multiple Sclerosis in Cerebral Spinal Fluid

Ms. Andrea Kreitz Summer 2009  
Emergent Patterns of Cytokine Expression in Chronic Fatigue Syndrome and Gulf War Illness

Ms. Christina Yang Summer 2009  
A Computational Study of Neuronal Migration in the Developing Neocortex  
Recipient of the Henry Anton Deutsch Medical Summer Research Award

Mr. Michael Gallagher 2008-2009  
A Computational Study of Neuronal Migration in the Developing Neocortex

Ms. Ann Aspler and Ms Carly Bolshin Summer 2007  
Evidence of Altered Neuroendocrine- immune Function in a Population-based Study of Chronic Fatigue Syndrome.

Mr. Bernhard Handle (senior year design project) 1995-1996  
Hydrometallurgical electro-refining of high-purity tellurium  
Supervisor: Dr. P. Paschen, Montan-University, Leoben, Austria

**Graduate Teaching.**

**MTH 6301** Statistical Design and Analysis of Experiments Winter 1995  
Dept. Appl. Mathematics and Industrial Engineering, École Polytechnique de Montréal  
Adjunct Professor, developed and taught graduate course linking linear regression theory to the construction and analysis of multivariable experimental studies.

**Graduate Supervision/ Co-supervision**

Paule Marceau (M.Sc.) - Industry Co-supervisor 1997-2001  
Modeling the impact of particle size distribution of mechanical properties of wood composites  
Supervisor : Dr. A. Cloutier (Université Laval)

Brendan Cote (M.Sc.) - Industry Co-supervisor 1997-2000  
Applicability of advanced computational networks to the modeling of complex geometry  
Supervisor: Dr. D. Thérien (McGill University)

Jean Sébastien Bolduc - Industry Co-supervisor 1996-1998  
Cellular-automata based nonlinear adaptive controllers  
Supervisor: Dr. D. Therein (McGill University)

Bodhana Ratitch - Industry Co-supervisor 1996-1998  
Continuous function identification with fuzzy cellular automata  
Supervisor: Dr. D. Thérien (McGill University)

Trevor Fenton - Industry Co-supervisor 1996-1997  
Finite element modeling of porous networks of composite materials  
Supervisor: Dr. H. Budman (University of Waterloo)

Timothy Knapp - Industry Co-supervisor 1996-1997  
Adaptive geometry neural network based control of chemical processes  
Supervisor: Dr. H. Budman (University of Waterloo)

**Postgraduate Teaching/ Supervision.****Supervision of post-doctoral research** 2007 – present

Mr. J Fuite (PhD 2007)  
A Network Theoretical Study of Neuro-immune Deficiency in Chronic Fatigue and Gulf war Illness.

**Supervision of research associate****2006 –2007**

Mr. Eric Carpenter  
Bi-stability in repair from injury and rejection of kidney allografts

**OTHER PROFESSIONAL AND SCHOLARLY ACTIVITIES**International Committees

Invited member July 2010  
International Panel for Canadian Consensus Document on ME/CFS

Invited member Jan. 2010  
US Veteran's Administration Planning Committee for Gulf War Illness Genome-wide Association Study (CSP #585)

Associate Editor, Mar. 2010  
BMC Systems Biology, BioMed Central, London, UK

Administrative and Research Committees

Member Aug. 2007 – present  
Department of Medicine Research Committee

Member Division of Pulmonary Medicine	July 2006 - present
Head Computational Biology Management Committee - Alberta Transplant Applied Genomics Centre	July 2006 - present
Voting member Scientific Management Executive Committee - Transplant Transcriptome Project Genome Alberta/ Genome Canada	Sept. 2006 - present
Member Faculty Committee for Review of the Institute for Biomolecular Design	Mar. 2007 - present
Member Faculty Committee for University Wireless Services	Oct. 2007 - present
<u>Student academic committees.</u>	
Member Ph.D. Committee – Mr. Zhipeng Cai Dept. of Computer Science, University of Alberta	Sept. 2006 - present
Member Ph.D. Committee – Mr. Anmmd Kamruzzaman School of Public Health, University of Alberta	Oct. 2006 - present
<u>Organising committees</u>	Nov. 2006 – June 2007
Organising Committee member Session Chair - Numerical Techniques for Creating Biological Insights “Omic” Technologies to Phenotype Disease: A Satellite Symposium of the 9th Banff Conference on Allograft Pathology, Edmonton, AB. Jun	

## **Appendix H: Mary Ann Fletcher Curriculum Vitae**

### **UNIVERSITY OF MIAMI Curriculum Vitae**

**Date:** January 28, 2010

#### **I. PERSONAL**

1. Name: Mary Ann Fletcher
2. Home Phone: 305-596-5535
3. Office Phone: 305243-6288
4. Home Address: 10700 SW 90<sup>th</sup> Ave, Miami, FL 33176
5. Current Academic Rank: Professor, tenured
6. Primary Department: Medicine
7. Secondary Appointments: Microbiology/Immunology; Psychology; Pediatrics, member of the Graduate Faculty
8. Citizenship: USA

#### **II. HIGHER EDUCATION**

1. Texas Technological College, B.S. (honors), 1959
2. University of Texas, M.A., 1961
3. Baylor University, Ph.D., 1966
4. Northwestern University, postdoctoral fellowship, 1966-68

#### **III. CERTIFICATIONS AND LICENSURES**

1. Diplomat American Board of Bioanalysis - High Complexity Laboratory Director, Clinical & Technical Consultant
2. State of Florida licensed and CLIA certified as Clinical Laboratory Director

#### **IV. EXPERIENCE**

1. 1981 - Present: Tenured Professor of Medicine, University of Miami Miller School of Medicine, Miami, FL
2. 1982 - Present: Professor of Microbiology/Immunology, UM Miami School of Medicine, Miami, FL
3. 1989 - Present: Professor of Psychology, UM, Coral Gables, FL
4. 1982 - Present: Director, E.M. Papper Clinical Immunology Laboratory, UM School of Medicine, Miami, FL
5. 1978 - Present: Member of the Graduate Faculty, UM, Coral Gables, FL
6. 1978 -1981: Associate Professor of Microbiology, UM School of Medicine, Miami, FL
7. 1977 - 1981: Tenured Associate Professor of Medicine, UM School of Medicine, Miami, FL
8. 1972 – 1976 : Assistant Professor of Medicine, UM School of Medicine, Miami, FL
9. 1970 - 1972: Adjunct Assistant Professor of Biology, Illinois Institute of Technology, Chicago, IL
10. 1969 -1972: Assistant Director, Division of Hematology, Michael Reese Hospital and Medical Center, Chicago, IL
11. 1969 -1972: Assistant Attending Physician, Special Staff, Michael Reese Hospital and Medical Center, Chicago, IL..
12. 1967 -1969: Instructor and Asst Professor, Dept. Microbiology, Northwestern University Medical School, Chicago, IL
13. 1966 - 1969: Postdoctoral fellow, Northwestern University, Evanston Hospital, Evanston, IL
14. 1963 - 1966: Research Associate, Graduate Research Institute of Baylor University, Dallas, TX

15. 1962 - 1963: Clinical Bacteriologist, Spohn Hospital & Driscoll Found. Hospital, Corpus Christi, TX
16. 1959 - 1961: Research Assistant, Dept Microbiology, Univ. of Texas, Southwestern Medical School, Dallas, TX.

## V. PUBLICATIONS (BOOKS AND MONOGRAPHS)

1. Springer, G.F., Fletcher, M.A. and Pavlovskis, O. Homogeneous Erythrocyte Glycoproteins with Blood Group -, Virus -, and Endotoxin Receptor Activities. In *Protides of the Biological Fluids* 15:109-122, 1967, H. Peeters, ed., Elsevier Pub. Co., Amsterdam.
2. Springer, G.F., Fletcher, M.A: Cell Surface Receptors: Structural Aspects and Importance in Immunopathology In *Organ Transplantation*, pp47-62, Hymer and Ricken, eds., Schattauer, Stuttgart, 1969.
3. Levey, G.S., Fletcher, M.A. and Klein, I. Glucagon and Adenylate Cyclase: Binding Studies and Requirements for Activation. In *Advances In Cyclic Nucleotide Research*, Vol. 5, edited by G.I. Drummon, P. Greengard, and G. Robinson, Raven Press, New York, 1975.
4. Levey, G., Fletcher, M.A and S. Ramachandran: Methods to Characterize the Cardiac Glucagon Receptor. In *Methods in Receptor Research*, edited by M. Blecker, pp. 143-147, Marcel Dekker, Inc., New York, 1976.
5. Fletcher, M.A, Lo, T.M. and Graves, W.R.: Glycoproteins from the Bovine Erythrocyte Membrane. *Glycoconjugate Research* Vol. II. *Proceedings of the Fourth International Symposium On Glycoconjugates*. R. Jeanloz., Academic Press, Inc. N.Y. pg. 1047-1050, 1978.
6. Fletcher, M.A., Lo, T.M. and Caldwell, K.E.: Interaction of Limulin with Membrane Glycoproteins, *Biomedical Applications of the Horseshoe Crab (Limulidae)* E. Cohen, ed., Alan Liss, Inc. N.Y. pg. 655-664, 1979.
7. Claflin, A.J., McKinney, E.C and Fletcher, M.A.: Immunologic Studies of Prostate Adenocarcinoma In Animal Models. *Model System For Prostate Cancer*, G. Murphy, ed., Alan Liss, Inc. N.Y., 365-376, 1980.
8. Saez, L., Latif, A., Caldwell, K. and Fletcher, M.A.: Heterophile Antigen Common to Human Tissues and Bovine Erythrocytes. in *HLA Antigens In Health, Aging and Malignancy*, (Cohen, E. and Singa, D.P., ed.) Alan R. Liss, Inc., New York, pp. 221-225, 1983.
9. Caldwell, K.E. and Fletcher, M.A A Paul-Bunnell Antibody Reactive Glycoprotein From Canine Erythrocyte Membranes. In *Glycoconjugates*, Davidson, E.A., Williams, J.C. and DiFerrante, N.M., eds., Prager, N.Y. p590-590, 1986.
10. Fletcher, M.A., Caldwell, K.E. and Klimas, N.G. The Use of Glycoprotein-Latex Conjugates in a Rapid Slide Test for Paul-Bunnell (P-B) Antibodies in Unabsorbed Sera. *Clinical Immunology* Pruzanski, W, Seligmann, M., eds, Elsevier Science Publ., Amsterdam, pg 247-250, 1987.
11. LaPerriere, A., Schneiderman, N., Antoni, A. and Fletcher, M.A. Aerobic Exercise Training and Psychoneuroimmunology in AIDS Research. in *Psychosocial Aspects of AIDS*. edited by L. Temoshok and A. Baum. Lawrence Erlbaum and Associates, 1990.
12. Klimas, N.G., Baron, G.C. and Fletcher, M.A. The Immunology of HIV-1 Infection. in *Stress, Coping and Disease* edited by Schneiderman, N., McCabe, P., Fields, T. and Skyler, J. Lawrence Erlbaum and Associates, Hillsdale, N.J. p193-209, 1991.
13. Fletcher, M.A., Klimas, N.G., Morgan, R. and Gjerset, G. Lymphocyte Proliferation Assays. in *Manual of Clinical Laboratory Immunology*, 4th edition, Rose, N. and Fahey, J. eds., Amer. Soc. Microbiology. pg. 213-219, 1992.
14. LaPerriere, A., Antoni, M., Klimas, N.G., Schneiderman, N. and Fletcher, M.A. Psychoimmunology and Stress Management in HIV-1 Infection. in *Update in Psychoneuroimmunology*, Gorman, J.M. and Kertzner, R.M., eds. *Progress in Psychiatry*, #35, Spiegel, D. series ed., American Psychiatric Press, Washington, D.C., p. 81-112, 1991.
15. LaPerriere, A., Antoni, M., Fletcher, M.A. and Schneiderman, N. Exercise and Health Maintenance in AIDS. In *Clinical Assessment and Treatment in HIV: Rehabilitation of a Chronic Illness*. Galantino, F., ed., Slack, Inc., Thorofare, NJ. pg.65 - 76, 1992.
16. Klimas, N.G., Morgan, R., Salvato, F., Flavia, Van Reil, Millon, C. and Fletcher, M.A. Chronic Fatigue Syndrome and Psychoneuroimmunology. In *Stress and Disease Progression: Perspectives in Behavioral Medicine*. Schneiderman, N., McCabe, P. and Baum, A., eds. Lawrence Erlbaum, Assoc., Hillsdale, NJ, P121-137, 1992.
17. Nash, M.S. and Fletcher, M.A., The Physiologic Perspective: Immune System, In. *Aging with Spinal Cord Injury*. Whiteneck, G.G., ed. Demos Publications, New York, pg. 159-181, 1992.

18. Antoni, M., Schneiderman, N., LaPerriere, A., Bourguinon, L. and Fletcher, M.A.. Psychoneuroimmunology and stress responses in HIV-1 seropositive and at-risk gay men. In *Stress and Disease Progression: Perspectives in Behavioral Medicine*. Schneiderman, N., McCabe, P. and Baum, A., eds. Lawrence Erlbaum, Assoc., Hillsdale, NJ, 139-163, 1992.
19. Antoni, M., Schneiderman, N., LaPerriere, A., O'Sullivan, M. and Fletcher, M.A.. Mothers with AIDS. in Ahmad, P. (ed). *Living and Dying with AIDS*. Plenum, N.Y. Pg. 45-86, 19
20. Klimas, N.G., Morgan, R., vanRiel, F.. and Fletcher, M.A., Clinical Observations Regarding Use of an Anti-Depressant, Fluoxetine. in Chronic Fatigue Syndrome, In *Chronic Fatigue Syndrome*, P. Goodnik and N.G. Klimas, eds., American Psychiatric Press, Washington, D.C., p95-108, 1993.
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22. Fletcher, M.A., Morgan, R. and Klimas, N.G. Immunologic Consequences of Treatment for Drug Abuse. In Friedman, H. et al., eds. *Drugs of Abuse, Immunity and AIDS*, Plenum Press, New York, p241-246, 1993.
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24. Klimas, N.G., Fletcher, M.A., Walling, J., Garcia-Morales, R., Patarca, R., Moody, D. and Ocarma, T. Ex Vivo Expansion, Activation and re-infusion in to Donor with rIL-2 - a Phase 1 Study. in *Retroviruses of Human AIDS and Related Diseases*, Girard, R, M. and Valette, L., eds, in 8th Colloque des Cent Gardes., pg. 285-290, 1992.
25. Antoni, M, Esterling, B, Lutgendorf, S, Fletcher, MA, and Schneiderman, N. Psychosocial Stressors, herpes virus reactivation and HIV infection. In Stein, M and Baum, A, eds. *AIDS and Oncology: Perspectives in Behavioral Medicine*. Erlbaum, Hillsdale, NJ, 1995.
26. Schneiderman, N., Antoni, M., Ironson, G., Klimas, N.G., LaPerriere, A., Kumar, M., Esterling, B. and Fletcher, M.A.. HIV-1, Immunity and Behavior. in *Handbook of Human Stress and Immunity*. ed by Glaser, R. and Kiecolt-Glaser. Academic Press, Inc. New York, 1995.
27. Goodkin, K., Blaney, N., Tuttle, R., Lehman, J. Feaster, D., Burkhalter, J., Leeds, B., Mahmood, R., Baum M., Kumar, M. and Fletcher, M.A. The psychoneuroimmunological impact of bereavement and a support group in HIV-1 infection. In Goodkin, K., ed. *Psychoneuroimmunology: Stress, Mental Disorders and Health*. American Psychiatric Press, Inc., Washington, D.C. , 1995.
28. Ironson, G., Antoni, M., Schneiderman, N., LaPerriere, A., Klimas, N. and Fletcher, M.A. Stress Management interventions and psychosocial predictors of progression in HIV-1 infection. in K. Goodkin, ed. *Psychoneuroimmunology, Stress, Mental Disorders and Health*. American Psychiatric Press, Washington, D.C., 1995.
29. Lutgendorf, S., Antoni, M., Schneiderman, N., Ironson, G. and Fletcher, M.A. Psychosocial interventions and quality of life changes across the HIV spectrum. in Baum, A. and Dimsdale, J., eds. *Perspectives in Behavioral Medicine*. Erlbaum, Hillsdale, N.J. 205-239, 1995.
30. Schneiderman, N, Antoni, M, Ironson, G, Lutgendorf, S, Hurwitz, B, Klimas, N, LaPerriere, A and Fletcher, MA. Psychoneuroimmunology und HIV/AIDS. In Schedolwski, M and Tewes, U, eds. *Psychoneuroimmunology*, Spektrum Akademischer Verlag, Germany, 577-633, 1996.
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32. Fletcher, M.A., Urban, R., Asthana, D., Walling, J., Friedlander, A. And J.B. Page. Lymphocyte proliferation in *Manual of Clinical Laboratory Immunology*, Rose, N., deMacario, E., Folds, J., Lane, H.C. and Nakamura, R., eds. ASM Press, Washington, D.C., p 313-319, 1997.
33. Patarca, R., Fletcher, M.A. and Podack, E. Cytolytic cell functions. in *Manual of Clinical Laboratory Immunology*, Rose, N., deMacario, E., Folds, J., Lane, H.C. and Nakamura, R., eds. ASM Press, Washington, D.C., p 296-303, 1997.
34. Patarca, R., Maher, K., Goodkin, K. and Fletcher, M.A. Cryopreservation of peripheral blood mononuclear cells in *Manual of Clinical Laboratory Immunology*, Rose, N., deMacario, E., Folds, J., Lane, H.C. and Nakamura, R., eds. ASM Press, Washington, D.C., p 281-286, 1997.
35. Fletcher, M.A., Ironson, G., Goodkin, K., Antoni, M., Schneiderman, N. and Klimas, M.A. Stress and immune function in HIV-1 disease. In Workman, E. and Hubbard, J., eds. *Stress Medicine*. CRC Press, Boca Raton, FL, p229-242, 1998.
36. Fletcher, MA, Klimas, NG and Patarca, R. Cytotoxic Lymphocytes, Fink, G., ed in *Encyclopedia of Stress*, Academic Press, pg. 639-643, 2000.

37. Ironson, G, Antoni, M, Schneiderman, N, LaPerrere, A, Klimas, N and Fletcher, MA. Stress management interventions and psychosocial predictors of progression in HIV-1 infections. In Goodkin, K (Ed) *Psychoneuroimmunology, stress, mental disorders and health*. American Psychiatric Press, Washington, DC pp 317-356, 2000.
38. Ironson, G, Antoni, M, Schneiderman, N, Chesney, M, O'Cleirigh, C, Balbin, E, Greenwood, D, Lutgendorf, S, LaPerrere, A, Klimas, N and Fletcher, MA. Coping: Interventions for optimal disease management in HIV. In Chesney, M and Antoni, M (Eds) *Innovative Approaches to Health Psychology: Prevention and Treatment Lessons from AIDS*. American Psychological Association, Washington, DC, 167-196. 2002.
39. Maher, K, Klimas, NG and Fletcher, MA. Immunology of Chronic Fatigue Syndrome. In *Handbook of Chronic Fatigue Syndrome*. Jason, Fennell and Taylor, eds John Wiley and Sons, 1-38, 2003.
40. Klimas, N, Fletcher, MA, Maher, K and Lawrence, R. Psychoneuroimmunology and Fatigue. In *Fatigue as a Window to the Brain*, DeLuca, J, ed., MIT Press, 281-298, 2005.
41. Fletcher, MA, Klimas NG, Cytotoxic Lymphocytes. G. Fink, ed.. In *Encyclopedia of Stress.*, 2<sup>nd</sup> edition, Academic Press, Oxford, 711-715, 2007

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1. Prager, M.D., Fletcher, M.A, and Efron, K.: Mechanisms of the Immunohematologic Effect of Papain and Related Enzymes. *J. Immunol.* 89:834,840, 1962.
2. Prager, M.D. and Fletcher, M.A: Mechanisms of the Effect of Trypsin on Specific Hemagglutination with Incomplete Anti-Rh Antisera. *Proc. Soc. Exptl. Biol. Med.* 111:722-725, 1962.
3. Prager, M.D. and Fletcher, M.A.: The Effect of Enzymatic Release of Sialic Acid From Human Erythrocytes on Rh Agglutinations. *J. Immunol.* 94:165-170, 1966.
4. Prager, M.D., Soules, M. and Fletcher, M.A: Further Studies On The Mechanism Of The Effect of Enzymes on Erythrocyte Serology with Special Reference to Pronase. *Transfusion* 8:220-225, 1968
5. Springer, G.F., Fletcher, M.A. and Pavlovskis, O.: Isolation and Charaterization of Homogeneous Erythrocyte Glycoproteins with Blood Group - Virus and Endotoxin - Receptor activities. *Transplantation* 6:674-675,1968.
6. Springer, G.F., Schwick, G. and Fletcher, M.A: The Relationship of the Influenza Virus Inhibitory Activity of Glycoproteins to their Molecular Size and Sialic Acid Content. *Proc. Nat'l Acad. Sci.* 64:634-641, 1969.
7. Fletcher, M.A and Woolfolk, B.J. Immunochemical Studies of Infectious Mononucleosis. I. Isolation and Characterization of Heterophile Antigens from Hemoglobin Free Stroma. *J. Immunol.* 107:842-853, 1971.
8. Fletcher, M.A and Woolfolk, B.J.: Immunochemical Studies of Infectious Mononucleosis II. Sodium Dodecyl Sulfate Gel Electrophoresis of Membrane Glycoprotein Antigens. *Biochim. Biophys. Acta.* 278:163:174,1972
9. Klein, I., Fletcher, M.A and Levey, G.S.: Evidence for a Dissociable Glucagon Binding Site In A Solubilized Preparation of Myocardial Adenylate Cyclase. *J. Biol. Chem.* 284:5552-5554, 1973.
10. Fletcher, M.A and Lo, T.M.: Immunochemical Studies of Infectious Mononucleosis III. Isolation and characterization of Heterophile Antibodies. *Clin. Exptl. Immunol.* 16:607-614, 1974.
11. Levey, G.S., Fletcher, M.A., Klein, I., Ruiz, E. and Schenk, A: Characterization of I-131 Glucagon-binding In a Solubilized Preparation of Cat Myocardial Adenylate Cyclase: Further Evidence for a Dissociable Receptor Site. *J. Biol. Chem.* 249: 2665-2673, 1974.
12. Fletcher, M.A and Lo, T.M.: Immunochemical Studies of Infectious Mononucleosis of Effect of Proteases on the Glycoprotein of Horse Erythrocytes. *Proc. Soc. Exptl. Biol. Med.* 145:1100-1105, 1974
13. Hunter, S.J., Fletcher, M.A. and Bush, C.A.: Molecular Weight of the Major Acidic Glycoprotein of Horse Erythrocyte Membrane. *Arch. Biochem. Biophys.*163: 581-588, 1974.
14. Pope, R.M., Fletcher, M.A., Mamby, A., and Shapiro, R.: Rheumatoid Arthritis Associated with Hyperviscosity Syndrome and Intermediate Complex Formation. *Arch Int. Med.* 135:281, 1975.
15. Fletcher, M.A and Lo, T.M.: Immunochemical Studies of Infectious Mononucleosis V. Isolation and Characterization of Glycoprotein from Goat Erythrocytes. *J. Immunol.* 117:722,1976.
16. Fletcher, M.A., Lo, T.M., Graves, W. and Robles, M.: Isolation of Plasma Membrane Glycoprotein from Bovine Thymocytes. *Biochim. Biophys. Acta.* 465 341-352,1977.
17. Fletcher M.A., Lo, T.M., Levey, B.A. and Graves, W.R.: Immunochemical Studies of Infectious Mononucleosis. VI. A Radioimmunoassay for the Detection of Infectious Mononucleosis Heterophile Antibody and Antigen. *J. Immunol. Meth.*14:51-58, 1977.
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1. Bovine Glycoproteins and Use in Diagnosing Infectious Mononucleosis. 4,460,694
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## VIII. PUBLISHED ABSTRACTS AND PRESENTATIONS

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## IX. FUNDED RESEARCH (LAST FIVE YEARS)

### **Active**

Immunologic Mechanisms, Biomarkers and Subsets in CFS

NIAID R01AI065723 (PI MA Fletcher) 12/1/06 – 11/30/11

Goal of this project is to determine the immunologic basis for CFS pathogenesis

Role: PI, 25% effort

The Use of Comprehensive Molecular Profiling with Network and Control in GWI

GW080152 DOD (PI N Klimas)

07/15/2009 - 06/14/2011

GWI patients will be studied using gene array technology and neuron-endocrine-immunologic profiling pre-post exercise challenge.

Role: co-PI, 20% effort

University of Miami Developmental Center for Aids Research (D-CFAR)

NIAID SB04 1P30AI073961 (PI S Pahwa) 2007 – 2010

Direct core immunology lab for DCFAR members, Serve on developmental grants committee.

Role: Core lab director, 2.5% effort

### **Completed**

Neuropeptide Y and dipeptidyl-peptidase IV (CD26) in chronic fatigue syndrome

NIAAA R21AA016635 (PI MA Fletcher) 9/30/06-5/31/09

Goal of this project to determine the relationship of neuropeptide Y and dipeptidyl-peptidase IV to natural killer cell cytotoxicity in CFS.

Role: PI, 20% effort

Psychobiological Processes and Health in HIV.

NIMH R01 (PI G. Ironson) 7/01/03-5/31/09

This grant examines psychological and biological (CTL, NK, cortisol) predictors of disease progression in HIV/AIDS.

Role: Col. 5% effort

Patterns of Gene Activation in Gulf War Illness and Chronic Fatigue Syndrome

VA Merit Review, (PI N Klimas). 11/04 -11/09

Male and Female GWI and CFS patients will be studied using gene array technology pre-post exercise challenge.

Role: Co-PI, 10% effort

Efficacy of an Emotional Exposure Intervention in HIV.

NCCAM R01 (PI G. Ironson) 7/01/03-6/30/08

This study investigates the efficacy of emotional exposure and depth processing through writing on well being in patients with HIV/AIDS.

Role: Co.I. 5% effort

HIV/HCV Co-infection: HAART and Pathophysiology

NIHLBI R01 (PI B Hurwitz) 7/31/04 to 8/31/07

This study looks at the interactions of HAART and co-infection with HCV on the pathophysiology of HIV/AIDS

Role: Co-I, 5% effort

Massage Benefits in HIV+ Children: Mechanisms of Action

NCCAM R01AT002689 (PI G. Shor-Posner) 01/01/05-03/31/07

The two-fold intent of the above proposal is to evaluate the impact of massage therapy on immune recovery, and to investigate a potential neuroimmune mechanism of massage action.

Role: Co.I. 10% effort,

Center for Multidisciplinary studies of CFS

NIAID U01- AI- 459940 (PI N Klimas) 8/1/00 to 7/31/05

This was a study of the modification of the stress response through a program of cognitive behavioral stress management and its effect on immune function in patients with chronic fatigue syndrome.

Role: Project Leader and Core Leader

Center for Psycho-Oncology Research

NCI IMO1-RR16587 (PI M Antoni) 10/1/99 to 9/30/05

The center was devoted to the mind/body interactions in patients with cancer.

Role: Core Leader

## X. PROFESSIONAL SOCIETIES

American Association of Immunologists; American Society for Microbiology; American Society for the Advancement of Science; Society for Complex Carbohydrates; Association of Women in Science; Society for Experimental Biology and Medicine; Society for Clinical Cytology; Association of Medical Laboratory Immunologists, Steering Committee, 1987; Clinical Immunology Society; PsychoNeuroimmunology Research Society, Scientific Affairs Committee - 1993 1995; American Association of Bioanalysts, Association of Clinical Chemists

## XI. HONORARY SOCIETIES

Phi Kappa Phi, Alpha Epsilon Delta, Alpha Lambda Delta, Sigma XI, Fellow - Academy for Behavioral Medicine Research

## **XII. CONSULTANTSHIPS**

National Cancer Institute (NCI), Tumor Immunology Review Committee 1980 - 1982

NCI - Clinical Cancer Program Project Review Committee Site Visit Participant, 1978 - 1984

National Science Foundation (NSF), Postdoctoral Fellowship Review Committee, 1979 - 1980

NSF Ad Hoc Review, 1979 - 1986

National Institutes of Health (NIH), Immunotechnology Special Review Committee, 1986-87

National Institute of Mental Health (NIMH), Psychoneuroimmunology Training Grants, Site Visitor, 1987 and Special Review Committee, 2004.

National Institute of Drug Abuse (NIDA), Site Visitor, 1988

National Heart, Lung and Blood Institute (NHLBI), Site Visitor, Special Review Committee 1989, 1993, 1998.

National Institute of Allergy and Infectious Diseases (NIAID), Special Review Committee, In vitro methods for AIDS Clinical Trials, 1995

NIAID, Ad Hoc reviewer for AIDS study section, 1991;

NIAID, Special Review Committee for Pediatric AIDS, 1995

NIAID, HIV Vaccine Development Review Committee, 1996, 1997, 1999, 2002 - 2005

NIAID, Special Review Committee for CFAR proposals, 2002, 2003

NIAID, HIV Special Emphasis Panel, 2004

NIH, CFS/FMS/TMD Review Panel, 2007, 2010

NIAID, Special Review Committee, 2007, 2008, 2010

## **XIII. EDITORIAL BOARDS AND REVIEWING**

Clinical Applications in Cytometry; Clinical and Diagnostic Immunology; Journal of Chronic Fatigue Syndrome; Clinical and Applied Immunology Reviews ; Section editor: ASM Manual of Medical Laboratory Immunology, 5th edition. Ad Hoc reviewer, FASEB, Natural Immunity, Annals Internal Medicine, Psychosomatic Medicine, Brain Behavior and Immunity, Journal Infectious Diseases

## **XIV. TEACHING**

Specialization: Immunology, Medical Laboratory Immunology, Psychoneuroimmunology

Mentor: Pre-doctoral trainees:

Martin Rosenthal, 2006-2008

Zackary Barnes, 2008-2009

Natalie Hone, 2009

Mentor - Ph.D. candidates:

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George Ann Baron, Ph.D. 1983 - 1987  
Brian Esterling, Ph.D. 1989 - 1991

Mentor – Post-doctoral fellows:

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Zuhair Latif, Ph.D. 1980-1983  
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Lisetti Said, M.D. 1980-1983  
Gerson Silveria, M.D. 1984-1985  
Olga Torres, M.D. 1985-1986  
Fernando Salvato, M.D. 1987-1989  
Roberto Patarca, Ph.D. M.D. 1990-1994  
Kevin Maher, Ph.D., 1993-1994  
Hector Pons, Ph.D., 1994-1995  
Maria Jose Miquez-Burbano, M.D. 1994-1996  
Desh Asthana, Ph.D., 1994 - 1997  
Denise Dixon, Ph.D., 1997 – 1999  
Lina Garcia, M.D., 2007-2009  
Maria Vera, 2008-2009

Thesis and Dissertation Committees:

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Tammy Enos 1998- 2001  
Steven Burke, 1999-2002  
Connor O'Cleirigh 2000 -2006  
Adam Carrico 2004 – 2007  
Orit Weitzman 2004  
Blake Scallop 2005 – 2007  
Corina Lopez 2009 -

## **XV. SCHOOL AND DEPARTMENTAL COMMITTEES AND OFFICES**

1. Medical School Council, 1976-1978. 2003-09
2. Faculty Senate Council Rep. to Medical School Council, 1979-1982.
3. School of Medicine Rep. to Consultative Committee for the Presidential Search, 1979-1981.
4. Admissions Committee, Ph.D.- M.D. 1974-1977.
5. Department of Medicine Research and Space Committee, 1978-1979.
6. Graduate School Faculty Screening Committee, 1980-1982, Chairperson, 1983
7. School of Medicine Rep. to Consultative Committee for Provost Search, 1982.
8. Scientific Advisory Committee, 1983-1985 (Chairperson); 1993-1996; 2002-07.
9. Appointment, Promotion and Tenure Committee, 1981-1984, 2007-10.
10. Institutional Self-Study, Faculty Review Subcommittee, Chairperson, 1986-1987.
11. Clinical Research Center - Protocol Review Committee, 1990-1992.
12. Center for AIDS Research - Steering Committee, 1989-1994.
13. Clinical Research Center, Executive Committee, 2008 -2009.
14. Scientific Review Committee for DCFAR Developmental Grant Applications. 2007 – 2009.

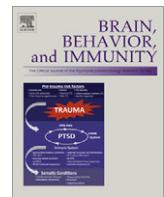
## **XVI. UNIVERSITY COMMITTEES & OFFICES**

1. Faculty Rep. to Presidential Search and Selection Committee, 1979-1981.
2. Vice-Chairperson, Faculty Senate, 1979-1981.
3. Faculty Senate, 1979-82; 2000-03, 2003-12.
4. Faculty Senate Council Representative from School of Medicine, 1979-1982.
5. Academic Planning Committee, 1976-1978.
6. Academic Personnel Board, 1979-1981, 1992-1994.
7. Faculty Senate Committee on Committees, 1979-1981.
8. Women's Commission, President, 1984-1986.
9. President's Ad Hoc Committee on Research and Development - Chairperson of Subcommittee on Academic Affairs, 1981-1982.
10. Intercollegiate Athletics Governing Board, 1981-1982.
11. Research Council, 1982-1985.
12. Search Committee for Vice-President for Research and Dean of the Graduate School, 1984-1985
13. Consultative Committee for V.P. for Research & Dean of Grad. School, 1984-1985 (Chair).
14. University Self Study - Committee for Intercollegiate Athletics, 1985-1986.
15. Faculty Senate Committee for Rank, Salary and Conditions of Employment, 1987-1990 (Chair).
16. Faculty Senate Professional Conduct Committee, 1992 -1994 (Chair).
17. Faculty Senate Athletic Committee, 1993 - 1997, Chair, 1993-1994.
18. Faculty Senate Committee on Academic Services, 1997-1999.
19. Faculty Senate Committee on Women and Minorities, 2000-2004 (Chair).
20. Faculty Senate Committee on Institutional Priorities, 2000-2001.
21. Faculty Senate General Welfare Committee, 2004-2011.
22. Faculty Senate Ad Hoc Committee on Academic Freedom, 2008-2009.
23. Faculty Senate Ad Hoc Committee on Evaluation of Sub Deans, 2008-2009.

## **XVII. COMMUNITY ACTIVITIES**

Health Crisis Network, Medical Advisory Committee, 1985

Women and AIDS Workshops, National Organization for Women, State and National Conferences, 1988, 1989, 1990, 1991, 1996, 1997.



## A pilot study of immune network remodeling under challenge in Gulf War Illness

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### ABSTRACT

Gulf War Illness (GWI) is a complex disorder affecting nervous, endocrine and immune regulation. Accordingly, we propose that GWI presents with a distinct pattern of immune signaling. To explore this we compared interaction patterns linking immune markers and their evolution during exercise. Blood was collected from 9 GWI and 11 control subjects prior to a Graded eXercise Test (GXT) ( $t_0$ ), at peak effort ( $t_1$ ) and 4 h post-exercise ( $t_2$ ). Salivary cortisol and plasma, serum or culture supernatants were analyzed for concentrations of neuropeptide Y (NPY), IL-1 $\alpha$ , IL-5, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$  and soluble CD26 (sCD26). Immune cell populations were surface stained for CD19, CD2, CD3, CD4, CD8, CD26, CD56, CD16, and CD11a. Mutual information (MI) networks linking these immune markers were generated in each group at each time point. Graph theory was used to describe the evolution of each network's structure and identify potential nucleating points. Distinct in topology, GWI networks had more abundant connections but were less organized. NPY, IL-1 $\alpha$ , TNF- $\alpha$  and CD2+/CD26+ nodes were better integrated in the GWI network at rest. Under effort ( $t_1$ ) these differences were replaced by significant restructuring around nodes for CD19+ B cell population, IL-5, IL-6 and soluble CD26 concentrations. This pattern subsided post-exercise. Further analysis indicated that IL-1 $\alpha$  and CD2+/CD26+ nodes strongly influenced this characteristic modulation of B and T cell network motifs. This potentially heightened lymphocyte and HPA axis responsiveness to IL-1 stimulation in the context of a mixed Th1:Th2 immune signature supports an autoimmune component in GWI etiology.

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### 1. Introduction

Within months after their return from Operation Desert Storm an alarming number of Gulf War veterans began to report a variety of symptoms, including fatigue, musculoskeletal discomfort, skin rashes, and cognitive dysfunction (Haley, 1997; Fukuda et al., 1998; Wolfe et al., 1998). Several models have been proposed linking these symptoms to the hazardous conditions encountered by servicemen including infectious agents, medical prophylaxis, depleted uranium as well as chemical and biological warfare agents, and the psychological stressors of combat (Fukuda et al., 1998; Rijkema et al., 2005; Gronseth, 2005). However these remain speculative and we still have no clear understanding of the pathophysiology of Gulf War Illness (GWI) nor do we have a characteristic biomarker for this illness. As the body is regulated by a

number of highly integrated systems, consideration of the basic physiology of response to stress whether psychological, chemical or other may provide a useful starting point. Indeed clinical presentation of this illness shares dimensions with that of another stress-mediated illness namely Chronic Fatigue Syndrome (CFS) (Kang et al., 2003; Eisen et al., 2005).

Activation of the "fight or flight" response has a broad and significant impact on the body including modulation of the function and status of the immune system (Silverman et al., 2005). Not surprisingly several facets of immune dysregulation have been reported in these subjects by members of our team (Maher et al., 2003) including seminal work describing a significant expansion in activated CD2+/CD26+ T cell population (Klimas et al., 1990). A major contributor to the regulation of CD4+ T, NK and NK T cells, CD26 also plays a major role in T cell-dependent antibody production and immunoglobulin isotype switching in B cells. Abnormal expression is found in autoimmune diseases, HIV-related illness and cancer. Increased CD20+/CD25+ and decreased CD20+/CD23+ B cell populations were observed in CFS by Robertson et al. (2005) however these trends did not achieve statistical significance ( $p < 0.01$ ). This was not the case for GWI patients where a significantly elevated CD19+ B cell population was reported along with

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increased concentration of auto-antibody directed against myelin basic protein (MBP) as well as striated and smooth muscle (Vojdani and Thrasher, 2004). In addition both CFS (Robertson et al., 2005) and GWI (Vojdani and Thrasher, 2004) patients also exhibited greater numbers of CD3–/CD16+ NK cells. CD25+/CD56+ and CD2–/CD56+ fractions also appeared higher in CFS but not with statistical significance. While NK cells may be greater in number we have found they display an impaired cytotoxicity (Fletcher et al., 2002; Siegel et al., 2006), a result confirmed by other groups (Vojdani and Thrasher, 2004). Indeed significantly reduced levels of intracellular perforin have been observed in CD3–/CD56+ NK and CD3+/CD8+ cytotoxic T cells in these veterans (Whistler et al., 2009).

Though frequently studied in isolation these immune cell subsets exist in the context of a larger and well-integrated community. It is reasonable to expect therefore that these anomalies in cell population would also manifest as altered patterns of cytokine signaling. Indeed veterans with CFS present with significantly higher levels of IL-2, IL-10, IFN- $\gamma$ , and TNF- $\alpha$  than non-fatigued veterans (Zhang et al., 1999). Similarly Skowera et al. (2004) reported significantly elevated levels of IL-2, IFN- $\gamma$  and IL-4 producing CD4+ cells in non-stimulated culture compared with asymptomatic veterans. Expression of IL-4 in these subjects was lost however after controlling for vaccination status, depression and atopic illness. In vitro polyclonal activation also revealed significantly increased levels of IL-10 producing memory CD4+ cells in symptomatic veterans. More recently members of our group reported concurrent expression of IFN- $\gamma$  with Th2 cytokine IL-5 in PHA-stimulated culture during the course of a standardized exercise challenge (Whistler et al., 2009). This would suggest a concurrent Th2 component and argue against conventional Th1 polarization.

Though elements of immune involvement in GWI and CFS have been reported, many of these reports are conflicting and the nature of this involvement remains unclear. Unfortunately these studies typically focused on a narrow segment of the diverse cell types that compose the immune community and the signals that coordinate their interaction (Maher et al., 2003). By the same token no attempt is made to cast individual molecular messages in the greater context of concurrent immune signaling and changing cellular demographics. Indeed, analysis of these biological systems as coordinated networks has received relatively little attention. Efforts have focused largely on the visual inspection of small assemblies of known pathway elements (Kerr et al., 2008a,b; Whistler et al., 2009). Only recently has graph theory been used to quantitatively describe broad shifts in molecular interaction across phenotypes (Emmert-Streib, 2007). We have since extended this work by defining methods for identifying individual elements (Fuite et al., 2008) and sub-assemblies (Broderick et al., 2010) driving illness-mediated shifts in network topology.

In this work we apply these graph theoretical methods to the integration and analysis of a broad spectrum of cellular and molecular markers describing the immune status of Gulf War veterans. Once again our hypothesis is that immune abnormalities in GWI like CFS (Broderick et al., 2010; Fuite et al., 2008) are characterized not only by persistent low-grade inflammation but also more importantly by changes in the patterns of immune signaling suggestive of a change in functional mode. In an extension of previous analyses we now propose a methodology for integrating multiple networks constructed at sequential time points along the course of an exercise challenge. Our results indicate that immune networks not only differ significantly between groups but that network structure in GWI is bulkier and potentially less efficient in its response to exercise challenge. The latter is characterized by a shift in the balance of Th1 and Th2 signals under effort suggestive of an auto-antibody driven immune cascade. The propagation of interactions across time suggests that this characteristic evolution

in network structure may be triggered or amplified by initial changes in IL-1 $\alpha$  and CD2+/CD26+ cell abundance. This result would not have emerged from a conventional analysis of expression levels. We emphasize that because of the limited number of subjects involved this remains a preliminary analysis and these findings are presented as potential focal points for further investigation.

## 2. Materials and methods

### 2.1. Sample collection and processing

#### 2.1.1. Cohort recruitment

As part of a larger ongoing study a subset of 10 GWI and 11 control subjects recruited from the Miami Veterans Administration Medical Center were used in the present analysis. Subjects were male and ranged in age between 30 and 55. Inclusion criteria was derived from Fukuda et al. (1998), and consisted in identifying veterans deployed to the theater of operations between August 8, 1990 and July 31, 1991, with one or more symptoms present after 6 months from at least 2 of the following: fatigue; mood and cognitive complaints; and musculoskeletal complaints. Subjects were in good health prior to 1990, and had no current exclusionary diagnoses (Reeves et al., 2003). Medications that could have impacted immune function were excluded. Use of the Fukuda definition in GWI is supported by Collins et al. (2002). Control subjects consisted of gulf war era sedentary veterans and were matched to GWI subjects by age, body mass index (BMI) and ethnicity.

*Ethics statement:* All subjects signed an informed consent approved by the Institutional Review Board of the University of Miami. Ethics review and approval for data analysis was also obtained by the IRB of the University of Alberta.

#### 2.1.2. Subject assessment

All subjects received a physical examination and medical history including the GWI symptom checklist as per the case definition. Psychometric questionnaires included the Multidimensional Fatigue Inventory (MFI) (Smets et al., 1995), a 20-item self-report instrument designed to measure fatigue, and the Medical Outcomes Study 36-item short-form survey (SF-36) (Ware and Sherbourne, 1992) assessing health-related quality of life. Results of these psychometric surveys may be found in additional file Appendix A (Table S7). With only one exception (physical functioning index), SF-36 scores were significantly lower and MFI scores significantly higher in GWI patients than in healthy controls ( $p < 0.05$ ).

Immune response was stimulated by administering a standard Graded eXercise Test (GXT) using a Vmax Spectra 29c Cardiopulmonary Exercise Testing Instrument, Sensor-Medics Ergoline 800 fully automated cycle ergometer, and SensorMedics Marquette MAX 1 Stress ECG. The McArdle protocol (McArdle et al., 2007) was used where subjects pedal at an initial output of 60 W for 2 min, followed by an increase of 30 W every 2 min until the subject reaches: (1) a plateau in maximal oxygen consumption (VO<sub>2</sub>); (2) a respiratory exchange ratio above 1.15; or (3) the subject stops the test. Prior to the exercise challenge a first blood draw was conducted subsequent to a 30-min rest. Second and third blood draws were conducted upon reaching peak effort (VO<sub>2</sub> max) and at 4 h post-exercise, respectively. Importantly, these assessments were conducted at the same time of day for all subjects to control for diurnal variations in cortisol and other similarly affected indicators.

#### 2.1.3. Laboratory analyses

At each blood draw five 8-mL tubes of blood were collected in CPT vacutainers (B-D-Biosciences, San Jose, CA). Heparinized whole blood was then cultured 48 h with phytohemagglutinin at 37 °C,

5% CO<sub>2</sub>. Following incubation, culture supernatants were collected and frozen at –70 °C until analyzed for cytokine content. Culture supernatants, serum or plasma for cytokine studies were aliquoted and stored at –70 °C and not thawed more than once. Concentrations of IL-1 $\alpha$ , IL-5, IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  were determined from blood following ex vivo stimulation with phytohemagglutinin (PHA) of supernatants in culture. Levels of IL-6, IL-10, and TNF- $\alpha$  were also determined in plasma. In all cases measurement was performed using commercial ELISA kits (Immunotech, Miami, FL), quality-controlled using National Institute for Biological Standards and Control/World Health Organization cytokine reference standards. Serum soluble CD26 (sCD26) was also measured by ELISA (Bender Med Systems, Burlingame, CA) and NPY was measured using a RIA from Alpco Diagnostics (Salem, NH). Salivary cortisol was determined by immunoassay using the Salimetrics high sensitivity kit (State College, PA).

Flow cytometry was performed on each sample to determine lymphocyte subset abundance using a Beckman/Coulter FC500. Whole blood samples were stained in 5 color combinations, with the appropriate concentrations of antibodies, erythrocytes lysed and the cells fixed with the Optilyse C reagent (Beckman-Coulter Corp., Hialeah, FL). Lymphocyte, monocyte and granulocyte populations were determined using light scatter and back gating on fluorescence for the CD45 bright and CD14 negative population. The isotype control served as reference for negative events. Spectral compensation was established daily. Quality control included optimization for lymphocyte recovery, purity of analysis gate, lymphosum, and replicate determined according to CDC guidelines. In addition, measurement of intracellular cytotoxic protein concentrations was performed using quantitative fluorescence. Levels of intracellular perforin, granzyme A or granzyme B, conjugated to phycoerythrin within lymphocytes subsets, both NK cells and CD8+ T cells, were simultaneously assessed with a 5-color system using a maximum-yield protocol (Maher et al., 2002). The median and the dispersion about the median for all immune markers at each phase within each group are listed in Table S2. Equivalent values for the mean and standard deviation may be found Table S3 of Appendix A (Supplemental Data).

## 2.2. Numerical analysis

The collection and analysis of biomarker data was conducted in four basic steps as described in Supplemental Fig. S1. First peripheral blood was collected at three time points during the course of a standard graded exercise challenge: prior to exercise ( $t_0$ ), at peak effort ( $t_1$ ) and 4 h post-exercise ( $t_2$ ) (Fig. S1, Step 1). Samples were analyzed as described in Section 2.1.3 and the expression levels of *individual* biomarkers were compared across patient groups at each time point (Fig. S1, Step 2). The next step and the focus of this work was to identify significant trends shared by pairs of biomarkers (Fig. S1, Step 3). To do this association networks were constructed for each group at each time point. These networks were then compared in terms of their general structure (Fig. S1, Step 4a) and regions of the networks examined for local shifts in topology that might be illness-specific (Fig. S1, Step 4b). The basic metrics used to describe global and local network structure are described conceptually in Supplemental Figs. S2 and S3, respectively. These will be described in mathematical detail in the following paragraphs. A general review of network theory and its applications in biology may be found in Barabási and Oltvai (2004) as well as in Huber et al. (2007).

Association networks were constructed using mutual information criteria (MI) as implemented in the ARACNe software (Margolin et al., 2006). The mutual information MI(X;Y) shared by variables X and Y corresponds to the total entropy  $H(X)$  and  $H(Y)$  of these variables minus their joint entropy  $H(X, Y)$  as defined in Eqs. (1)–(3). The null probability of each MI value was com-

puted by sub-sampling the data with replacement. Networks for the GWI and non-fatigued control groups were generated separately from a consensus of 50 sub-sampled networks. In our previous work, MI networks constructed from similar data were stable over a wide range of cut-off  $p$ -values (Fuite et al., 2008). As a result a threshold significance of  $p \leq 0.001$  for MI values was chosen for this analysis. This consensus averaging across sub-sampled data sets and the fact that MI assigns equal influence to each measured value makes this approach quite robust to outliers (Craddock et al., 2006; Butte and Kohane, 2000). For additional detail we have included the values for conventional Spearman rank-based cross-correlation of immune markers within each group at every phase of exercise in Tables S4–S6 of Appendix A (Supplemental Data):

$$H(X) = - \sum_{i=1}^n p(x_i) \log(p(x_i)) \quad (1)$$

$$H(X, Y) = - \sum_{j=1}^n \sum_{k=1}^m p(x_j, y_k) \log(p(x_j, y_k)) \quad (2)$$

$$MI(X; Y) = H(X) + H(Y) - H(X, Y) \quad (3)$$

Indirect associations were removed using data processing inequality (DPI). DPI states that if variables X and Z interact only through a third variable Y and no alternative path exists between X and Z, then the least of the three MI values ( $X \leftrightarrow Y$ ,  $Y \leftrightarrow Z$ ,  $X \leftrightarrow Z$ ) can only result from an indirect interaction and should be disregarded.

General topological differences in networks were evaluated using graph edit distance (Bunke, 2000) generalized for continuously weighted graphs. The graph edit distance is based on the minimum summed “cost” involved in removing and inserting graph edges to transform one network into another. The distance implied is proportional to the magnitude of the weight change in each edge. The weighted graph edit distance,  $d_{GED}$ , between two undirected networks of order  $N$  with adjacency matrices,  $A$  and  $B$ , can be described as follows:

$$d_{GED} = \sum_{i=1}^N \sum_{j \geq i}^N |a_{ij} - b_{ij}| \quad (4)$$

Significance of edit distance was estimated by estimating a null distribution of edit distance based on a set of reference networks identified by random sub-sampling of non-fatigued subjects.

Local restructuring of network features that drive these global changes in topology was described in terms of centrality or connectedness. First, node centrality or the direct connectivity of each node  $i$  to its immediate neighborhood  $N_i$  was computed as  $\sum_{j \in N_i} M_{ij}$ . Second, eigenvector centrality was also computed as a measure of connectedness of an individual node to its second neighbors. Eigenvector centrality  $x_i$  will increase when a node  $i$  is linked directly to other nodes that are well connected to the rest of the network. Eigenvector centrality values  $x_i$  were adjusted for differences network size based on the intuitive notion that a “star network” represents a structure of maximal centralization. Accordingly  $x_i \in [0, 1]$  and the maximum eigenvalue centrality  $x_i = 1$ , only occurs if the node is at the center of a star network (Ruhnau, 2000).

Beyond the characterization of individual nodes, the extent of centralization for the network as a whole was compared to that of a “star network” of similar size and order. For any network with order,  $N$ , and normalized principle eigenvector,  $\hat{X}$  (with a maximum component,  $x_{\max} = \max[x_i]$ ), a graph-eigenvector-centralization index,  $C_{\text{eigenvector}}$ , is given by:

$$C_{\text{eigenvector}} = \frac{\sum_{i=1}^N (x_{\max} - x_i)}{\sum_{i=1}^N (1 - x_i)} \in [0, 1] \quad (5)$$

Node degree centrality provides indication of each node's immediate membership and influence within a network. However we have

available 3 networks describing association patterns at sequential points in time. To exploit this we defined a metric  $E_i(t)$  we call the *node extent* for node  $i$  and time  $t$  as a cumulative measure describing how each node's influence propagates through these sequential networks. This is calculated recursively for each node  $i$  at time  $t$  by adding the immediate node degree to the node degree of all first neighbor nodes  $j$  connected to node  $i$  at time  $t+1$  (Eq. (6)). The extent  $E_i(t)$  of node  $i$  at time  $t$  thus provides a measure of the magnitude of cumulative influence each node exerts in the overall network across time. As we expect the strength of association to dissipate over time we constrain the definition such that any two nodes in a given extent sub-network will be linked by a path length less than or equal to the maximum network MI. This maximum value  $MI_{max}$  is an upper bound equivalent to the MI shared by a node with itself:

$$E_i(t) = \Omega_i + \sum_{j \in N_i^t > t} M_{ij} E_j \quad \text{where} \quad \Omega_i = \sum_{j \in N_i^t} M_{ij} \quad (6)$$

Graphical rendering was performed using a “spring-electrical” embedding (Pemmaraju and Skiena, 2003) where nodes are idealized as similarly charged objects that repel each other by virtue of a global electric force. Links are imagined as springs with spring-constants, proportional to their MI weights. Such representations tend to cluster highly connected nodes while spreading weakly linked nodes (Fuite et al., 2008).

The reader should note that stringent significance thresholds have been applied to the identification of network edges in this exploratory work (Fuite et al., 2008). This was done to maximize network stability and minimize false discovery. These safeguards are not without penalty and may result in reduced network coverage. Indeed recent work by Altay and Emmert-Streib (2010) confirms that ARACNe is conservative in its identification of edges and produces very low false positive rates. These authors also report that ARACNe like its peers only identifies approximately one third of all underlying interactions. Of these suppressive interactions are generally favored. However with ARACNe and MRNET, this selection bias was undetectable in small cohorts such as the one used here.

### 3. Results

#### 3.1. Differential expression of molecular signal and immune cell abundance

A standard Mann–Whitney non-parametric test was used to compare median expression of each immune marker at each time point. Similarly the non-parametric Friedman test was used to evaluate trends in median expression over time. Although results presented in Table S1 show a general over-expression of cytokines in the GWI group only a subset of these achieve statistical significance. We observed significantly higher response to PHA stimulation in GWI subjects for IL-5 at all 3 times ( $p \leq 0.001$ ) and in IFN- $\gamma$  during and after challenge ( $p = 0.02, 0.02$ ). Higher TNF- $\alpha$  responsiveness is also observed ( $p = 0.05$ ) but only at rest ( $t_0$ ). This is accompanied by significantly higher concentrations of plasma IL-6 throughout the challenge ( $p \leq 0.03$ ). Interestingly increased levels of plasma IL-6 in GWI subjects did not appear to be offset by compensatory responses in salivary cortisol or IL-10 expression at the resolution offered by this cohort size. Indeed, cytokines that displayed a trend detectable in controls at this level of resolution were IL-1 $\alpha$ , IL-5 and IL-10 in culture as well as IL-10 and NPY in plasma ( $p \leq 0.05$ ). Of these only IL-5 in culture produced a significant trend in the GWI group ( $p = 0.03$ ).

Results in Table S1 also indicate that GWI subjects show a consistent deficiency at virtually all time points as well as significant

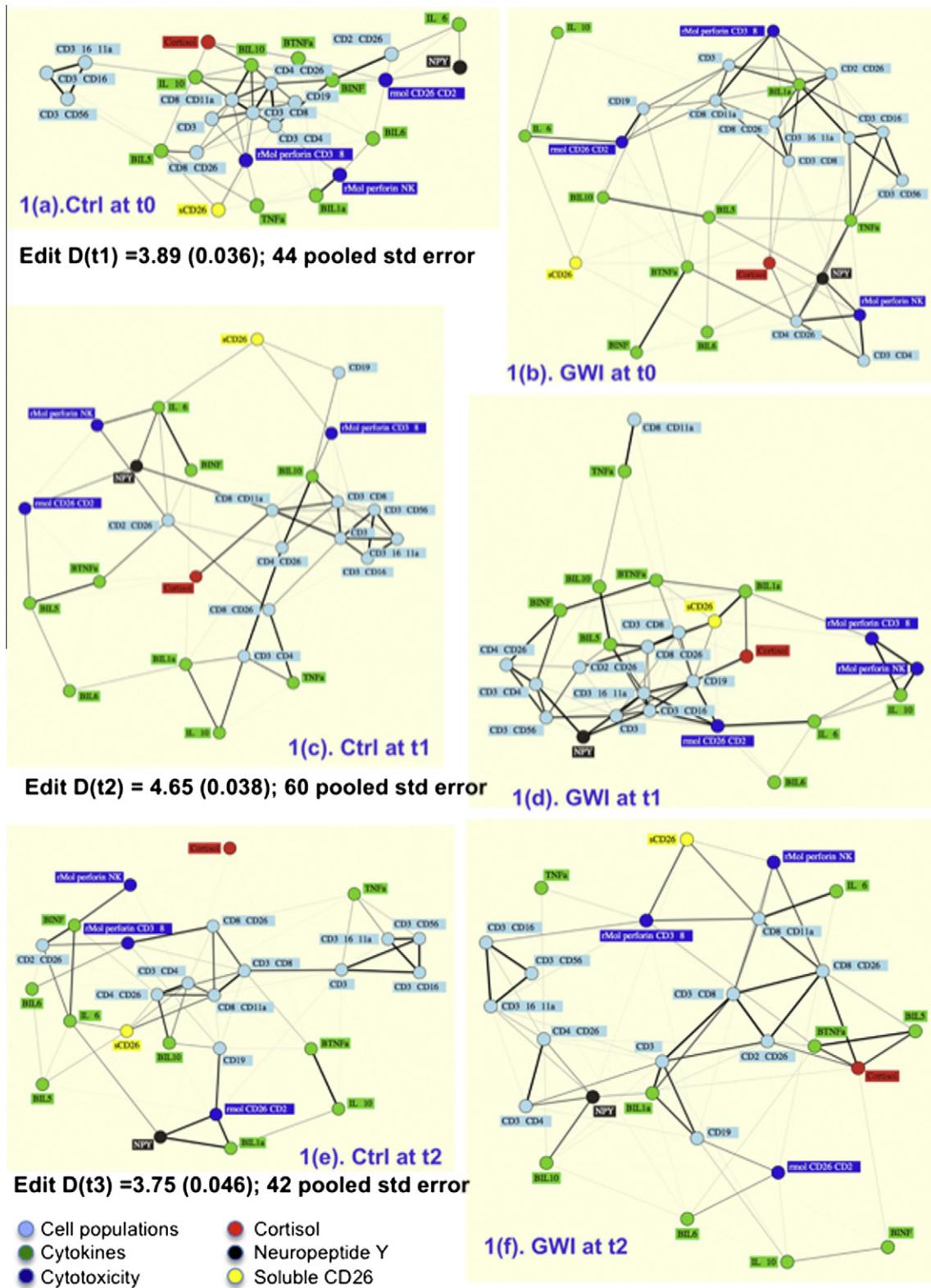
trends in the relative abundance of CD3-/CD56+, CD3-/CD16+ and CD3-/CD16+/CD11a+ NK cells. In contrast the relative abundance of CD2+ and CD8+ T lymphocytes expressing CD26/DPP-IV was elevated at rest and at peak effort in GWI subjects. Levels were still elevated at 4 h post-exercise but with marginal statistical significance ( $p = 0.11, 0.08$ , respectively). Once again these displayed significant trends with time ( $p \leq 0.02$ ). Interestingly CD4+/CD26+ cell abundance remained similar for both subject groups throughout the course of the exercise challenge. We also computed helper:suppressor T lymphocyte ratio at each time point using 2 different pairs of lymphocyte subsets. While differences in the CD3+/CD4+:CD3+/CD8+ ratio were not discernable with this sample size, we nonetheless found significant reductions in the ratio of CD4+/CD26+ to CD8+/CD26+ lymphocytes in GWI subjects both at rest and at peak effort. Both helper:suppressor ratios exhibited significant trends over time based on the Friedman test. Finally, no significant differences in intracellular cytotoxin expression were observed with the exception perhaps of intracellular perforin concentration in NK cells being marginally decreased at peak effort ( $p = 0.08$ ) compared to the relatively stable levels observed in controls.

#### 3.2. Patterns of immune cell coordinated activity

To isolate characteristic patterns of coordinated immune activity we constructed co-expression networks using mutual information (MI) as a robust measure of association between markers of immune cell abundance, intracellular cytotoxin concentration and levels of extracellular signal. Separate networks were identified for GWI and healthy controls at each of the three phases of the exercise challenge (Fig. 1). These networks were visibly different in architecture with GWI networks containing more abundant connections but being less organized in structure than control networks. Moreover GWI networks were separated from control group networks by statistically significant graph edit distances at all three time points. Networks were separated at rest by a graph edit distance of  $D_e(t) = 3.89$  or 44 times the standard error ( $p = 0.036$ ). This distance increased to 4.65 at peak effort (60 times the standard error;  $p = 0.038$ ) only to return to a value of 3.75 post-exercise (42 times the standard error;  $p = 0.046$ ).

Part of this separation in structure is due to the emergence of new associations. Results in Fig. 2 indicate that the immune network in healthy controls maintained roughly the same graph size, or the same total number of associations, throughout the exercise challenge. In contrast the network for GWI subjects at rest was populated by a much larger number of connections than in controls. GWI network size only increased further at peak effort. However post-exercise the network shed associations reducing its overall size to that of the control network. In a similar fashion the control network also maintained a relatively constant centrality index changing only minimally in the distribution and size of hub nodes. In comparison network centrality index was much lower for GWI at rest. Therefore not only were connections more abundant in GWI they were also more evenly distributed than in the control network prior to exercise. This changed under effort however as the GWI network underwent a redistribution of associations increasing in centrality index to match that of the control network. The corresponding increase in network size at peak effort indicates that formation of new hub nodes appears to be driven at least initially by the addition of new associations. A subsequent pruning of associations allowed this higher centrality index to be maintained post-exercise with a much smaller GWI network.

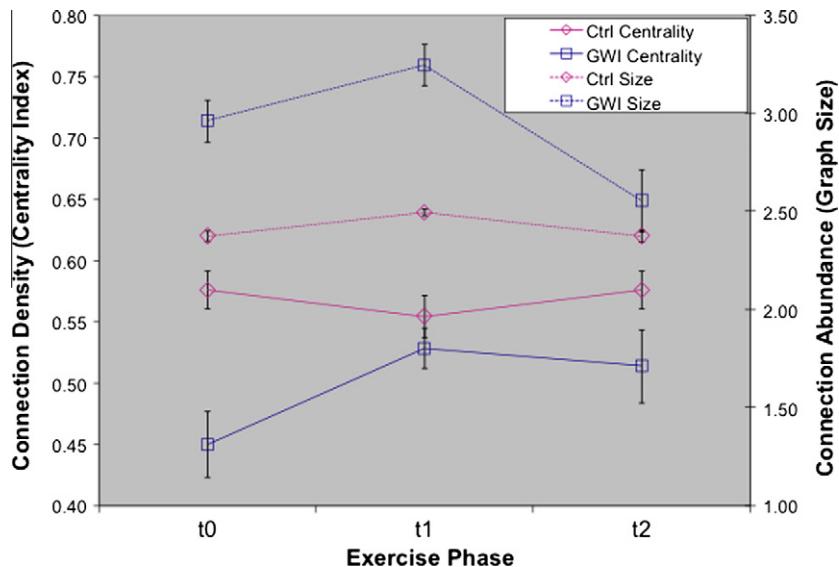
To identify areas of the immune network where most of this redistribution occurred we computed changes in node degree centrality and eigenvector centrality at each immune marker. Node degree centrality is an indicator of direct connectivity to neighbor-



**Fig. 1.** Immune marker association patterns differ significantly in GWI. Interaction networks based on mutual information (MI) linking the relative abundance of immune cell sub-types (light blue nodes) and the concentrations of cytokines (green nodes), cortisol (red node) and neuropeptide Y (black node) as well as intracellular cytotoxins (dark blue nodes). Networks are constructed at rest ( $t_0$ ), at peak effort ( $t_1$ ), and post-exercise ( $t_2$ ) for GWI and control groups. Line thickness is proportional to the strength of association and all associations are significant at  $p < 0.001$ . For each time point  $t_i$  the weighted graph edit distance  $D(t_i)$  separating the GWI from the associated control network is reported with its pooled standard error (SD).

ing nodes whereas eigenvector centrality is an indicator of indirect connectivity to the greater network community. Results presented

in Fig. 3 show significant changes occurred in the connectivity of several immune nodes. In particular nodes representing concentra-



**Fig. 2.** Exercise induces larger more diffuse immune response networks in GWI. The overall number of node–node connections (network size) and the network's dependency on hubs (centrality index) of interaction networks constructed for GWI and healthy control groups at rest ( $t_0$ ), at peak effort ( $t_1$ ), and post-exercise ( $t_2$ ).

tions of NPY, TNF- $\alpha$  and PHA-stimulated IL-1 $\alpha$  show dramatically higher local and global connectivity in GWI subjects at rest. Significant but more modest differences in indirect connectivity are also observed for plasma IL-6, relative abundance of CD2+/CD26+ cells and to a lesser extent CD3-/CD16+, CD3-/CD16+/CD11a+ and CD3-/CD56+ NK cells. Interestingly these differences in local connectivity dissipate at peak effort ( $t_1$ ) only to be replaced by significant restructuring of the GWI network around nodes for CD19+ B cell relative abundance, PHA-stimulated IL-5, IL-6 and soluble CD26 concentrations (sCD26). This pattern of connectivity is largely resolved at 4 h post-exercise ( $t_2$ ) and the GWI network resumes a structure similar to that observed initially at rest. Indeed the CD2+/CD26+ population had resumed much of its pre-exercise association at  $t_2$ . However nodes for relative abundance of CD3-/CD56+ and CD3-/CD16+ NK cells that were well connected to the network at rest still had not been re-instated 4 h after exercise.

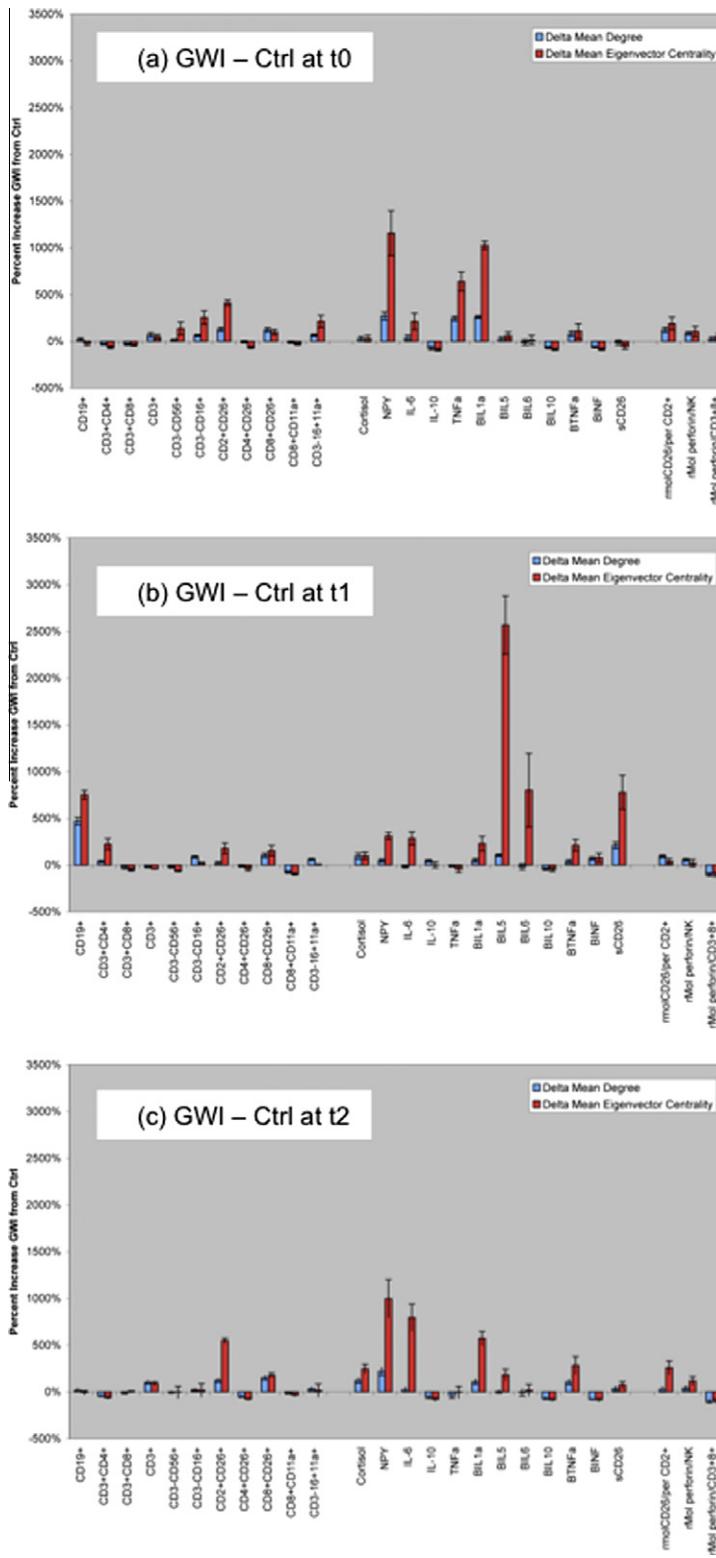
### 3.3. Propagation of influence

The importance of network remodeling occurring at each node across time can be summarized by the initial network extent  $E$  (Table 1). Results again show that in GWI change in network structure ( $p \leq 0.001$ ) during exercise challenge is widespread with virtually all immune nodes showing significant differences in extent  $E$  across groups. In terms molecular signal IL-1 $\alpha$  showed the most dramatic increase in GWI, followed to a lesser extent by IL-5 and TNF- $\alpha$  response in vitro. Conversely plasma IL-6 displayed the greatest deficit in extent followed closely by IL-10 both in plasma and in vitro. The sub-network of immediate neighbors associated with IL-1 $\alpha$  as it spans across the duration of the challenge is shown in Fig. 4a. Indeed in healthy controls IL-1 $\alpha$  at  $t_0$  is linked at  $t_1$  only to cortisol and at  $t_2$  only to PHA-stimulated IFN- $\gamma$ , and to a lesser extent CD2+/CD26+ and plasma IL-6. In GWI changes in IL-1 $\alpha$  are directly distributed to a much larger sub-network at maximal effort ( $t_1$ ): one that includes cortisol and a near complete complement of cytotoxic T and NK cell subsets. This sub-network remains large even 4 h post-exercise ( $t_2$ ). Significant differences in node extent ( $p < 0.001$ ) were also observed for most cell subsets with the exception of the CD3-/CD16+/CD11a+ NK sub-population. In general terms T, T helper and NK lymphocytes were less effective in propagating their influence and displayed decreased

extent in GWI with the largest loss being incurred by the CD3+/CD4+ T cell subset. In contrast nodes for cytotoxic T cell abundance and function were more influential with the largest of these gains occurring at nodes related to CD2+/CD26+ cell abundance and their surface expression of CD26 (rMol CD26 per CD2+). Increased node extent was also observed for the CD19+ B cell subset in GWI. Once again the first neighbor sub-network for CD2+/CD26+ shown in Fig. 4b increased in size dramatically during the course of the challenge in GWI. Indeed in the control network this node was associated mainly with its own future value at  $t_1$  and  $t_2$ , with minor associations to PHA-stimulated IFN- $\gamma$ , TNF- $\alpha$ , plasma IL-6 and NK intracellular perforin. In comparison the CD2+/CD26+ node in GWI was associated extensively with virtually all cytotoxic T and NK cell subsets under challenge and during recovery. This same massive recruitment of new associations under challenge was observed for relative CD26 expression by CD2+ cells.

## 4. Discussion

In this study, we generated an immune response to exercise and applied concepts from graph theory to investigate potential changes in immune signaling in GWI. Though our results appear to align well with elements of immune hypersensitivity (Münz et al., 2009) the overall number of subjects remains relatively small and this analysis should be considered preliminary. Also while the panel of cellular and molecular markers used was broader than most it remains relatively narrow and by no means constitutes a complete survey of immune response. These limitations notwithstanding we observed a mixed Th1/Th2 phenotype in GWI with significant elevations in both IFN- $\gamma$  and IL-5 production in vitro as well as pro-inflammatory IL-6 in plasma consistent with a recent review of available data (Peakman et al., 2006). Interestingly trends in IL-1 $\alpha$  and IL-10 response in culture as well as NPY in plasma were observed in controls but not in GWI subjects raising the possibility of some form of immune exhaustion. Innate senescence (Ongrádi and Kövesdi, 2010; Stout and Suttles, 2005) resulting in dampened IL-1 response has been linked to chronic stress (Bauer, 2008) and persistent inflammation (Prelog et al., 2008). Chronic inflammation has also been associated with IFN- $\gamma$  mediated inhibition of IL-10 expression (Conzelmann et al., 2010).



**Fig. 3.** GWI immune networks shift balance from Th1 motifs at rest to Th2 motifs under stress. Connected sub-networks linking node neighborhoods contributing to the overall extent at initial time  $t_0$  for (a) cytokine node IL-1 $\alpha$  and (b) cell abundance node CD2+/CD26+ in both GWI and healthy control subjects.

These changes coincided with significant deficits in NK cell abundance. Acute exercise normally stimulates NK cell activity in part due to the increased catecholamine levels and the high expression of  $\beta$ -adrenoreceptors on these cells (Jonsdottir, 2000; Richlin et al., 2004). Instead we found the baseline and amplitude of NK response significantly depressed in GWI. Diminished NK cell

activity has been induced by prolonged exposure to severe physical and psychological stress (Pedersen and Ullum, 1994; Gomez-Merino et al., 2005) and associated with the central nervous system's (CNS) opioid response (Jonsdottir, 2000). Interestingly NPY, a known mediator of this response (Saurer et al., 2008), was a central feature in the GWI immune networks. This loss in NK response was

**Table 1**

Median value (and scatter) of node extent  $E_i(t_0)$  at the outset of the challenge in GWI and healthy control subjects and significance of differences in extent between groups.  $E_i(t_0)$  is a measure of relative impact of an initial change of state in each cytokine, cell abundance and cytotoxin node as it carries forward through the challenge.

	Ctrl ( $t_0$ )	GWI ( $t_1$ )	$E_{\text{GWI}} - E_{\text{ctrl}}$	<i>p</i> -Value
<b>Signal molecules<sup>a</sup></b>				
<i>Saliva</i>				
Cortisol	3.12 (0.15) <sup>d</sup>	6.10 (0.32)	2.98	0.00
<i>PHA-stimulated blood culture</i>				
IL-1 $\alpha$	2.12 (0.12)	22.01 (1.68)	19.89	0.00
IL-5	3.39 (0.28)	13.06 (0.45)	9.67	0.00
IL-6	2.19 (0.18)	7.09 (0.46)	4.90	0.00
IL-10	11.75 (0.97)	6.88 (0.62)	-4.87	0.00
TNF- $\alpha$	2.00 (0.15)	10.23 (0.43)	8.23	0.00
IFN- $\gamma$	10.16 (0.44)	10.33 (0.88)	0.16	0.76
<i>Plasma</i>				
IL-6	12.60 (0.76)	3.45 (0.23)	-9.15	0.00
IL-10	10.07 (0.85)	3.78 (0.19)	-6.29	0.00
TNF- $\alpha$	0.05 (0.00)	4.89 (0.19)	4.84	0.00
SCD26	2.13 (0.08)	6.94 (0.53)	4.81	0.00
NPY	7.71 (0.55)	12.23 (0.84)	4.52	0.00
<b>Cell subset<sup>b</sup></b>				
<i>B cells</i>				
CD19+ %	3.63 (0.20)	8.40 (0.42)	4.78	0.00
<i>T lymphocytes</i>				
CD3+CD4+ %	11.03 (0.45)	2.45 (0.16)	-8.58	0.00
CD3+CD8+ %	16.55 (1.24)	12.59 (0.49)	-3.96	0.00
CD3+ %	12.70 (0.91)	15.35 (1.37)	2.65	0.01
<i>NK cells</i>				
CD3–CD56+ %	6.59 (0.24)	3.39 (0.12)	-3.19	0.00
CD3–CD16+ %	7.60 (0.51)	4.70 (0.26)	-2.91	0.00
CD3–16+ IIa+ %	2.59 (0.12)	2.95 (0.16)	0.37	0.18
<i>Cytotoxic and helper T cells</i>				
CD2+CD26+ %	6.34 (0.46)	20.11 (1.69)	13.77	0.00
CD4+CD26+ %	16.93 (0.56)	12.64 (0.62)	-4.28	0.00
CD8+CD26+ %	6.63 (0.48)	8.70 (0.69)	2.07	0.01
CD8+CDIIa+ %	14.79 (0.70)	19.43 (1.56)	4.64	0.00
<b>Cytotoxicity<sup>c</sup></b>				
rMol CD26 per CD2+	2.55 (0.12)	16.67 (0.73)	14.12	0.00
rMol perforin/NK	1.14 (0.08)	5.39 (0.40)	4.25	0.00
rMol perforin/CD3+CD8+	8.09 (0.65)	13.10 (0.81)	5.01	0.00

<sup>a</sup> Cytokines are reported in pg/10<sup>5</sup> lymphocyte cells.

<sup>b</sup> Cell abundance values are expressed as 10<sup>5</sup> lymphocyte cells.

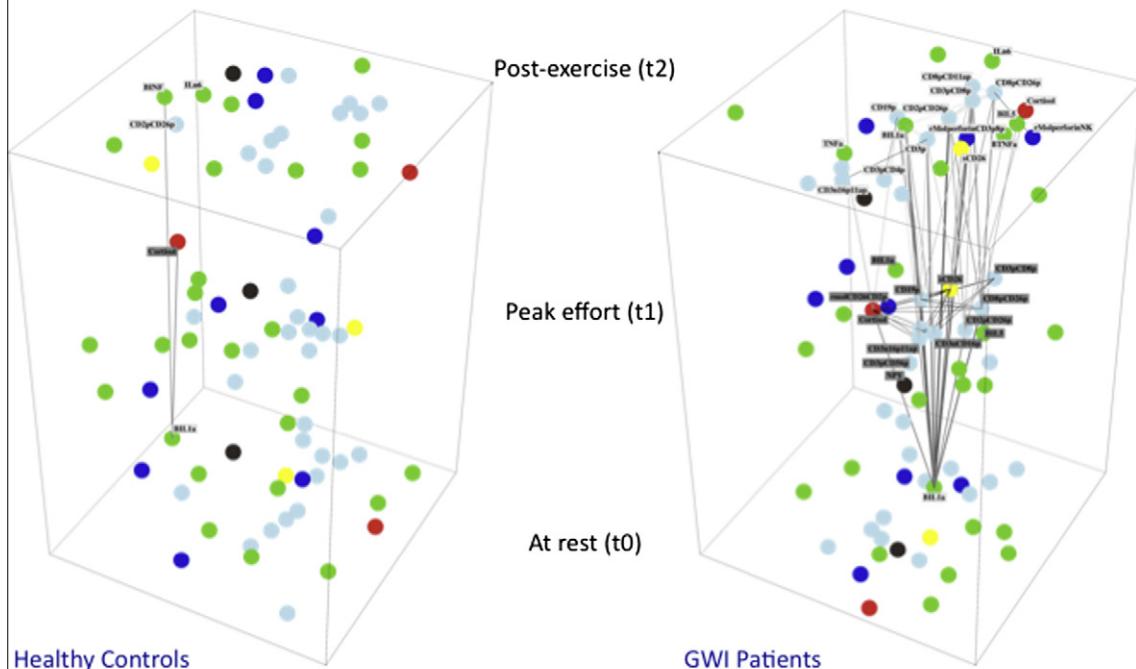
<sup>c</sup> Average relative number of cytotoxin molecules (rMol) per cell (Maher et al., 2002).

<sup>d</sup> Median absolute deviation from median MAD =  $|x - \bar{X}^*|$  and  $\bar{X}^*$  is the median value.

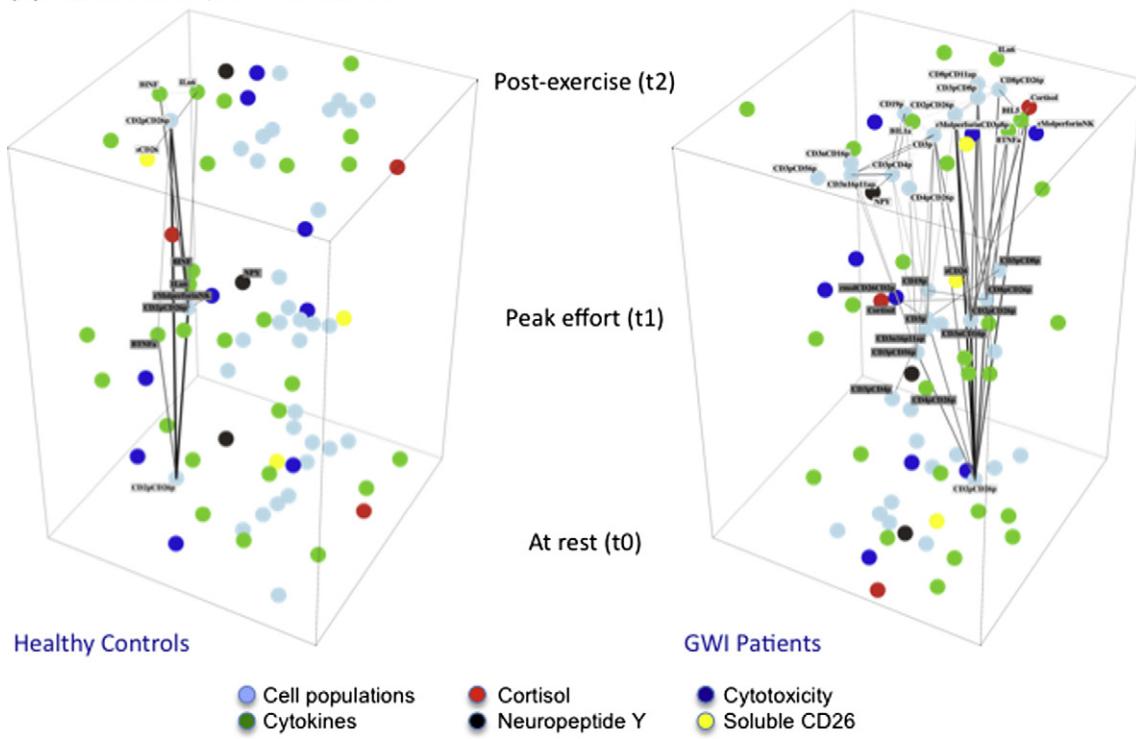
accompanied by persistent elevations in CD2+/CD26+ and CD8+/CD26+ T cell populations (Gleeson and Bishop, 2005; Klimas et al., 1990; Zhang et al., 1999; Raciatti et al., 2001; Whistler et al., 2009). Contrary to Vojdani and Thrasher (2004), we also found a significantly lower ratio of helper to suppressor (CD4:CD8) T cells in GWI that we suspect is due to our use of a narrower lymphocyte subset co-expressing CD26 (Reinhold et al., 2002). Interestingly, reduced CD4+:CD8+ ratios and elevated IL-5 also occur in autoimmune thyroid disease (Gopalakrishnan et al., 2010), an association previously noted in CFS by our team (Fuite et al., 2008).

A quantitative analysis of how these changes were orchestrated by the immune system revealed highly significant differences in associations between markers at all phases of exercise. Statistics indicated that the GWI immune network maintained a significantly higher number of active associations even at rest. Physical effort only prompted a disproportionate recruitment of even more immune interactions (Fig. 2). This suggests a re-routing of normal immune signaling to secondary response mechanisms in GWI and a less efficient use of immune resources. At the center of this inefficiency we found NPY, plasma TNF- $\alpha$  and PHA-stimulated IL-1 $\alpha$ , IL-6 and soluble CD26. Though not differentially expressed across groups, significant network restructuring occurred at these nodes.

Indeed connectivity of plasma NPY, TNF- $\alpha$ , and in vitro IL-1 $\alpha$  nodes to the greater network was especially elevated in GWI at rest. The same was true for nodes representing relative abundance of CD2+/CD26+ T, CD3–/CD16+ NK and to a lesser extent CD3–/CD56+ NK cells. At peak effort this preferential connectivity dissipated, only to emerge again in the recovery phase (Fig. 3) with the exception of TNF- $\alpha$  and NK cell nodes. The latter remained unrecovered 4 h post-exercise. This suggests that immune processes involving cytotoxic T and NK cell populations, active at baseline in GWI, may be disrupted at least temporarily by normal responses to exercise such as the release of cortisol. Interestingly a strong association emerges at peak effort in GWI between cortisol and IL-1 $\alpha$ , a known modulator of hypothalamic-pituitary-adrenal (HPA) function (Schmidt et al., 2003; Shintani et al., 1995; Buller et al., 2001) (Fig. 1d). Exercise also promoted dramatic increases in GWI network structure around nodes for IL-5 and IL-6 supernatant response, soluble CD26 and CD19+ B cell abundance. B cells are responsive to exercise (Richlin et al., 2004), causing up regulation of antibodies under certain conditions (Suzuki and Tagami, 2005). Accordingly IL-5 is a known promoter of B cell growth and immunoglobulin secretion while CD26 is involved in T cell-dependent antibody production and the transition to IgG and IgE

(a) Node IL-1 $\alpha$  expression in PHA-stimulated culture

## (b) Node % CD2+/26+ cell subset



**Fig. 4.** Small changes in initial CD2+/CD26+ abundance and IL-1 $\alpha$  concentration propagate more readily in GWI. Connected sub-networks link the first neighbor associations that emerge from the propagation across time of small changes in (a) IL-1 $\alpha$  and (b) CD2+/CD26+ abundance at initial time t(0) in both GWI and healthy control subjects. The strength of the illustrated associations contributed directly to the calculation of overall node extent exerted at t(0) for these markers.

antibody profiles associated with immune hypersensitivity (Yan et al., 2003). Increases in IgG auto-antibody levels have been reported in GWI (Vojdani and Thrasher, 2004) as have elevated levels of IgE (Klausermeyer et al., 1998). Interestingly chronic stress and inflammation can lead to a form of immune senescence that is

accompanied by increased auto-antibody production (Prelog, 2006; Weyand et al., 2003) and a lower affinity in humoral response (Johnson and Cambier, 2004). Our group has also reported evidence of altered CD19+ B cell status in CFS (Aspler et al., 2008) but with the notable absence of an active Th1 response

(Broderick et al., 2010). This transitory shift from Th1 to Th2 network motifs under challenge may constitute a signature of GWI homeostasis.

In an effort to identify potential nucleating points in these immune cascades we used a novel metric, node extent, to quantify the cumulated influence exerted by a node on the network as it propagates forward through time. We found that the majority of immune markers exhibited significant differences in node extent across patient groups. GWI was characterized by dramatic increases in the downstream influence of supernatant IL-1 $\alpha$  and IL-5 production with almost equally dramatic reductions in the propagation of plasma IL-6 and IL-10 effects. Similarly initial CD2+/CD26+ T cell abundance and CD26 surface expression contribute significantly to the evolution of network structure in GWI, followed to a lesser extent by B and other cytotoxic T cell subsets. In contrast, T helper and NK cell subsets were largely disassociated from network remodeling in GWI. Of all these candidate catalysts, inflammatory cytokine IL-1 $\alpha$  remains by far the most influential exhibiting an increase in node extent almost 4 times the average. IL-1 is a key player in several host defense mechanisms that extend beyond immune modulation alone to include a regulatory role in the neuronal (Dantzer et al., 2008) and endocrine systems (Schmidt et al., 2003; Shintani et al., 1995; Buller et al., 2001). Indeed IL-1 like IL-6 is a mediator of opioid receptor expression (Kraus, 2009) and this analysis suggests possible involved in an opioid-induced suppression of NK cell activity in GWI.

As an immune mediator IL-1 signaling has been shown to stimulate production of IFN- $\gamma$  and IL-17. Accordingly, imbalances in the IL-1/IL-1R/IL-1Ra system have been linked to the activation of auto-antigen specific T cells (Matsuki et al., 2006; Ishigame et al., 2006) and the acquisition of Th1 function in dendritic cells (DC). Such activity is consistent with CD26 surface expression as the latter is involved in modulating T cell proliferation via the CD2 and CD3 signaling pathways (Gorrell et al., 2001). Accordingly we observed strong interactions linking IL-1 $\alpha$  with CD8+/CD26+ and CD2+/CD26+ nodes in GWI at rest but not in healthy controls (Fig. 1a and b). This association pattern was disrupted during exercise possibly by the normal release of IL-1R antagonist (IL-1Ra). During this phase we observed strong associations with cortisol and soluble CD26 suggesting concurrent stimulation of B cell activity, possibly the release of auto-antigens, driven by exercise-induced damage. From a metabolic perspective increased IL-1 signaling has been associated with hyper-responsiveness to insulin and a significantly dampened response to glucose (Matsuki et al., 2003). IL-1 signal transduction also induces IL-6 (Orjalo et al., 2009). Like IL-1, the latter can increase insulin-stimulated glucose uptake (Pedersen, 2007) and serves as a possible indicator of insufficient access to blood glucose (Pedersen et al., 2007). Consequently our observations of elevated plasma IL-6 and the heightened role of IL-1 $\alpha$  in the GWI immune networks could point to poor insulin response in this group although additional data was not available to confirm this.

In this preliminary analysis we observed elements of chronic inflammation, possible senescence of innate immune components and a mixed Th1:Th2 cytokine signature. Elevated IL-6 and in vitro IFN- $\gamma$  combined depressed IL-1 $\alpha$  and IL-10 responsiveness point to chronic inflammation and possible premature immune senescence (Stout and Suttles, 2005; Conzelmann et al., 2010); risk factors in autoimmunity (Prelog et al., 2008). Abhorrent NK (Hammond and Kronenberg, 2003) and B cell activity (Yanaba et al., 2008) suggested by this data, both play a role in autoimmune illness. Finally, non-conventional mixed Th2/Th1 response profiles can be induced by sensitization with a foreign antigen mimicking self (Bünder et al., 2004). Likewise human proteins similar in structure to exogenous allergens can cross-react with allergen-specific IgE antibodies to induce sustained inflammation and lymphocyte

proliferation (Valenta et al., 2009; Mittermann et al., 2008; Aichberger et al., 2005). Such proliferation is consistent with the observed disengagement of NK cell activity (Wu and van Kaer, 2009), a subset shown to prevent activation of auto-reactive T cells in type-1 diabetes (Linsen et al., 2005) and of auto-reactive B cells (Wermeling et al., 2010) in lupus. IL-1 is a known promoter, both direct and indirect, of these auto-antigen specific immune cells (Matsuki et al., 2006) however we did not observe an over-expression of this cytokine. Instead we found that responsiveness to normal levels of IL-1 signaling may be amplified by the IL-6 rich environment (Renauld et al., 1989) in GWI thereby triggering and sustaining the activation of auto-reactive T and B cells in these subjects. Though these cytokines figure prominently as treatment targets (Hoffman, 2009) they are also common to a broad spectrum of inflammatory illnesses making them rather non-specific. Instead of considering these indicators in isolation, we propose that their patterns of interaction with the larger community of immune markers can produce a much more characteristic illness signature. While IL-1 $\alpha$  was identified as a possible nucleating point it is the cascade of interactions itself that constitutes a potential marker of GWI. Interventions would be directed at modulating component interactions, possibly by targeting receptors in specific immune cell subsets.

Based on these preliminary observations it would appear at least reasonable to include for future scrutiny autoimmune activity as a component of GWI. Of note, many of these elements would not have emerged from a conventional analysis of expression levels emphasizing that the latter offers a limited perspective of complex disorders like GWI. As many current network discovery tools share similar performance characteristics (Altay and Emmert-Streib, 2010), confirmation of these results should include the use of fundamentally different pathway-recognition algorithms (Efroni et al., 2007) and an integrative analysis of complementary biological data from different levels of signal transduction, for example transcriptional and post-transcriptional levels. Similarly the choice of immune stimulants will progressively favor a more selective interrogation of immune signaling and response mechanisms.

## Authors' contributions

Conceived and designed the experiments: M.A.F., N.G.K., and G.B. Performed the experiments: M.A.F. and N.G.K. Analyzed the data: J.F., A.K., and G.B. Contributed reagents/materials/analysis tools: M.A.F., A.K., G.B., and J.F. Wrote the paper: G.B., M.A.F., A.K., J.F., S.D.V., and N.G.K.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.10.011.

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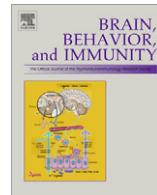
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## Reply to Letter to the Editor

**Circadian rhythms in cytokine secretion in chronic fatigue syndrome**

Dear Dr. Carlo-Stella,

Thank you for your interest in our research and your kind words of encouragement. We completely agree with your comments regarding the importance of circadian rhythm and sleep abnormalities in chronic fatigue syndrome (CFS). Indeed in one of our early efforts as a group we found that heart rate variability (HRV) during sleep was a key clinical variable that aligned strongly with characteristic gene expression patterns in distinguishing female CFS patients from control subjects (Broderick et al., 2006). Thank you also for raising the issue of altered regulatory dynamics in CFS. This has in fact been one of our basic models for describing CFS and similar complex illnesses such as Gulf War syndrome (GWS). In recent work we used a computational model to show that the stress response axis can support more than one set of regulatory dynamics by virtue of its very design (Ben-Zvi et al., 2009). As a result we certainly agree that ignoring the dynamics of homeostatic regulation and diurnal cycles can lead to erroneous interpretations. In an attempt to avoid this pitfall we controlled for circadian rhythm by conducting patient assessment and sample collection at the same time of day for all subjects throughout the study. Had funding been available to conduct a time course study we would have pursued this avenue enthusiastically. While static snapshots, even synchronized, do not offer a complete description of illness processes they can nonetheless deliver important insight if considered in the proper context. We believe the current article to be a valuable first step in establishing such context. The notion of context is implicit in the analysis of co-expression patterns and delivers insight unavailable from the conventional comparison of individual cytokine concentrations. Indeed several key network nodes were associated with cytokines that did not change in expression across patient groups. One could also argue that since these networks describe associa-

tions they necessarily incorporate some of the information from trends in cytokine expression. Admittedly this information would apply only to a narrow window in time. In the end a formal time course study, preferably one that spans two or more days, will be required to provide a complete description of illness-related changes in circadian dynamics. In keeping with this, much of our ongoing work is focused on novel methods for describing how these association networks change in structure over time as well as across patient groups. We are currently developing such methods using concepts rooted in network theory and applying them to time course data describing immune and endocrine response to exercise in CFS and GWS. Initial results are encouraging and we hope to report these shortly.

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# Biomarkers in Chronic Fatigue Syndrome: Evaluation of Natural Killer Cell Function and Dipeptidyl Peptidase IV/CD26

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## Abstract

**Background:** Chronic Fatigue Syndrome (CFS) studies from our laboratory and others described decreased natural killer cell cytotoxicity (NKCC) and elevated proportion of lymphocytes expressing the activation marker, dipeptidyl peptidase IV (DPPIV) also known as CD26. However, neither these assays nor other laboratory tests are widely accepted for the diagnosis or prognosis of CFS. This study sought to determine if NKCC or DPPIV/CD26 have diagnostic accuracy for CFS.

**Methods/Results:** Subjects included female and male CFS cases and healthy controls. NK cell function was measured with a bioassay, using K562 cells and <sup>51</sup>Cr release. Lymphocyte associated DPPIV/CD26 was assayed by qualitative and quantitative flow cytometry. Serum DPPIV/CD26 was measured by ELISA. Analysis by receiver operating characteristic (ROC) curve assessed biomarker potential. Cytotoxic function of NK cells for 176 CFS subjects was significantly lower than in the 230 controls. According to ROC analysis, NKCC was a good predictor of CFS status. There was no significant difference in NK cell counts between cases and controls. Percent CD2+ lymphocytes (T cells and NK cells) positive for DPPIV/C26 was elevated in CFS cases, but there was a decrease in the number of molecules (rMol) of DPPIV/C26 expressed on T cells and NK cells and a decrease in the soluble form of the enzyme in serum. Analyses by ROC curves indicated that all three measurements of DPPIV/CD26 demonstrated potential as biomarkers for CFS. None of the DPPIV/C26 assays were significantly correlated with NKCC.

**Conclusions:** By ROC analysis, NKCC and three methods of measuring DPPIV/C26 examined in this study had potential as biomarkers for CFS. Of these, NKCC, %CD2+CD26+ lymphocytes and rMol CD26/CD2+ lymphocyte, required flow cytometry, fresh blood and access to a high complexity laboratory. Soluble DPPIV/C26 in serum is done with a standard ELISA assay, or with other soluble factors in a multiplex type of ELISA. Dipeptidyl peptidase IV on lymphocytes or in serum was not predictive of NKCC suggesting that these should be considered as non-redundant biomarkers. Abnormalities in DPPIV/CD26 and in NK cell function have particular relevance to the possible role of infection in the initiation and/or the persistence of CFS.

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## Introduction

Chronic Fatigue Syndrome (CFS) is characterized by persistent and unexplained fatigue resulting in severe impairment in daily function and is defined by symptoms, disability, and exclusion of medical and psychiatric conditions that could explain the fatigue [1,2]. Population-based studies estimated the prevalence of CFS at 0.23% to 0.41% [3,4]. Costs to the US economy were estimated at \$9 billion in lost productivity and up to \$24 billion dollars in health care expenditures annually [5–7]. Complications and co-morbidity can be severe. For example, CFS was associated with chronic or

episodic cardiovascular and autonomic dysfunction [8]. Recent results from our group demonstrated reduced stroke volume and cardiac output in more severely afflicted CFS patients [9]. Reports suggested increased risk of cancer as well as suicide [10,11]. CFS affects all ethnic groups and socio-economic strata of society though at least 2 to 4 times as many women as men suffer from this illness [3,12,13]. Diagnosis using the case definition [1] requires the exclusion of any other medical explanation for these symptoms, yielding an inefficient, slow, error prone process. This is also costly because current clinical diagnosis typically involves tertiary care specialists.

Like many chronic illnesses CFS pathophysiology is complex and affects several of the body's main regulatory systems. There is a considerable literature describing immune dysfunction in CFS [14–16], although reviews of the immunology of CFS noted that universal agreement of immunological abnormalities had not been achieved, in no small part due to differences in methodologies, case definition and study quality [17,18]. However, redundant reports support 1) reduced function of natural killer (NK) cells [14,19] with deficiencies of perforin and granzymes in both NK cells and CD8 T cells [20]; 2) inflammation [21,22]; 3) altered cytokine profiles [9,10] with elevation of proinflammatory cytokines [11,12] and Th2 (T helper cell type 2) polarization [11,13]; and 4) chronic lymphocyte activation [14,16].

Current research efforts are directed toward identifying an individual marker or combination of markers sufficiently associated with CFS to facilitate objective diagnosis and management of CFS. Previously we reported that CFS patients with poor NK function had more fatigue, less vigor, more daytime dysfunction, and more cognitive impairment. Those results provided preliminary evidence in support of using NKCC as subgroup marker for disease severity in CFS [23].

Present on the surface of many cells including lymphocytes, DPPIV/CD26 is a transmembrane glycoprotein and a serine peptidase that spits proline dipeptides from the N-terminus of polypeptides, including chemokines and neuropeptides. An enzymatically active soluble form is found in serum. We have observed an elevated proportion of lymphocytes expressing this activation marker in CFS patients as compared to controls [14].

No widely accepted laboratory tests are available for the diagnosis or prognosis of CFS. This study sought to determine the accuracy by which measurements of NKCC or DPPIV/CD26 distinguished between subjects with the clinically derived diagnosis of CFS and matched healthy controls.

## Methods

### Objectives

Prior work indicated defective NK cell function and a high percent of T cells and NK cells expressing the activation marker DPPIV/CD26 in CFS cases. The aim of this study was to determine the potential of NKCC and DPPIV/CD26 as biomarkers for CFS.

### Participants

Chronic fatigue syndrome patients (age 18 to 60, mean age 44; 83% female) were drawn from the University of Miami Miller School of Medicine CFS and Immunodeficiency Clinic after they were diagnosed with CFS using the CDC clinical diagnostic

criteria [1,2] (Table 1). All were participants in research studies (NIH, Chronic Fatigue and Immunodeficiency Syndrome Association (CFIDS) or University of Miami). Exclusion criteria included any active medical condition that could explain the presence of chronic fatigue, including diabetes, the current use of immunomodulatory or antibiotic medications, and a past or present psychiatric diagnosis of psychosis (e.g., schizophrenia), dementia, major depressive disorder with psychotic or melancholic features, bipolar disorder, anorexia or bulimia nervosa, or alcohol/substance abuse within two years of the onset of the fatigue or anytime thereafter. The CFS subjects were studied at 2 to 25 years after onset of symptoms, with an average onset of 10 years. Healthy controls (Table 1) (age 23–74, mean age 41, 86% female) were drawn from University of Miami, NIH or CFIDS funded studies. Each completed a medical and psychiatric history that included medications and alcohol/substance abuse. Those with active medical or psychiatric conditions, immunomodulating medications or alcohol/substance abuse were excluded.

### Description of Procedures or Investigations Undertaken

**Blood Collection.** Morning blood samples were collected. For lymphocyte function assays and flow cytometry, sodium heparin tubes were used. The samples were held at room temperature and delivered to the laboratory within 4 hours. For complete blood counts, the blood was collected into ethylene diamine tetra acetic acid and delivered to the laboratory within 4 hours. Serum was separated from blood clot within 4 hours of collection into red stopper tube and stored at  $-20^{\circ}\text{C}$  until assayed.

**Natural killer cell cytotoxicity.** The bioassay for NKCC was performed using whole blood within 8 hours of collection in a chromium release assay as previously described [24]. The NK sensitive erythroleukemic K562 cell line was used as the target cell. The assay was done in triplicate at four target-to-effector cell ratios with 4-hour incubation. The % cytotoxicity at each target-to-effector ratio and number of CD3-CD56+ (NK) cells per unit of blood was used to express the results as % cytotoxicity at a target-to-effector cell ratio of 1:1.

**Determination of Lymphocyte Subsets and Assessment of Cell Surface Protein Concentrations by Quantitative Fluorescence.** For the assessment of lymphocyte subsets, and the quantitative fluorescence intensity studies of cell surface antigen, a whole blood lysis method was used [25]. Whole blood samples were stained in 4 color combinations, with optimized (saturating) concentrations of antibodies, erythrocytes were lysed and the cell fixed with the Optilyse C reagent (Beckman-Coulter Corp., Hialeah, FL). Determination of lymphocyte, monocyte and granulocyte populations was determined using light scatter and back gating on fluorescence for the CD45 bright and CD14

**Table 1.** Natural killer cell cytotoxicity and dipeptidyl peptidase IV/CD26 in chronic fatigue syndrome cases<sup>a</sup> compared to controls<sup>b</sup>.

Variable	Number of CFS Cases	Median (25–75 <sup>th</sup> percentile)	Number of Healthy Controls	Median (25–75 <sup>th</sup> percentile)	p
NKCC%	176	12 (8–21)	230	28 (20–37)	.000
% CD26+CD2+ Cells	75	61 (55–66)	100	52 (47–59)	.000
sCD26 in Serum (ng/ml)	73	489 (396–643)	122	671 (496–871)	.000
rMol CD26/CD2+ Cell	77	3625 (2844–4633)	102	4388 (3600–5388)	.001

<sup>a</sup>>80% female, average age 48;

<sup>b</sup>>80% female, average age 47.

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negative population using a Beckman Coulter multiparameter flow cytometer. The isotype control was the reference for negative events. Spectral compensation was established daily. Quality control included optimization for lymphocyte recovery, purity of gate of analysis, lymphosum, and replicate determinations. Phycoerythrin (PE) labeled antibodies were used for quantitative fluorescence determinations and the median fluorescence intensity value was entered into a least squares linear regression equation derived from analysis of the QuantiBrite fluorescence intensity standards (Beckton Dickenson, San Jose, CA). This permitted conversion from fluorescence intensity values to median numbers of molecules PE bound per cell (relative numbers of molecules protein expressed per cell at saturating concentrations of antibody; rMol/cell). This technique allowed us to determine the relative (r) number of molecules (Mol) of CD26 on CD2+ lymphocytes (T cells and NK cells) (Figure S1).

**Assay of Soluble CD26.** Soluble CD26 in serum was assayed with an ELISA kit from Bender MedSystems (Vienna, Austria). This assay has a sensitivity of 7.26 ng/ml and precision of 4.6%.

**Ethical issues.** All subjects signed an informed consent approved by the University of Miami Institutional Review Board. Participants were English speaking with at least an 8<sup>th</sup> grade education to ensure they were able to comprehend the informed consent as well as read and complete the questionnaires.

**Statistical Methods.** The nonparametric Mann-Whitney test was used to determine the magnitudes of between-group differences. The nonparametric Spearman test was used to determine correlations. Values of  $p < 0.05$  were considered statistically significant. The diagnostic accuracy of biomarkers was assessed in terms of true positive (sensitivity) versus true negative (1-specificity) using nonparametric receiver operating characteristics (ROC) analyses [26] available in the Statistical Package for Social Sciences (SPSS) software for Windows (SPSS Inc, Chicago, IL). The nonparametric ROC plot uses all of the data, makes no parametric assumptions and provides unbiased estimates of sensitivity and specificity, indicating the ability of a test to discriminate between two alternate states of health, in this case, CFS cases and healthy controls. The calculation of the area under the curve (AUC) provides a convenient single number. An  $AUC > 0.5$  indicates that the test shows no difference between the two groups while  $AUC = 1.0$  is found if the test gives a perfect separation between groups. The coordinates of the curves (COC), which provide the entire spectrum of sensitivity/specificity pairs and a complete picture of test accuracy, are given in Supplementary Files for each ROC plot.

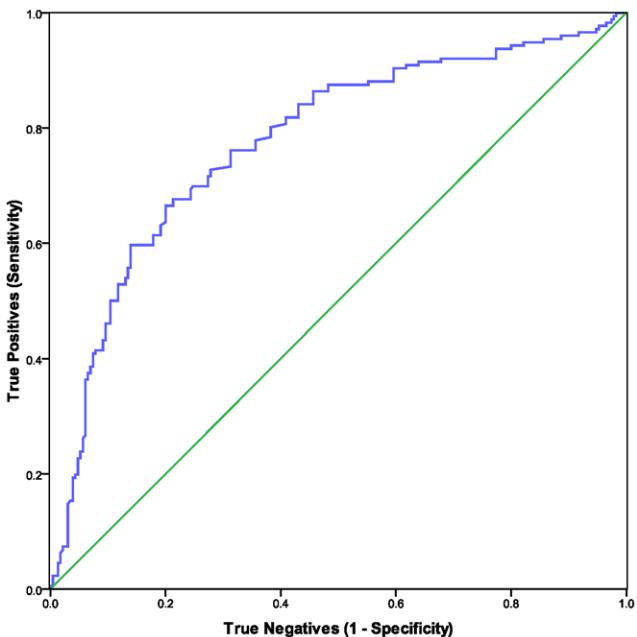
## Results

### Natural killer cell cytotoxicity

The NKCC values were significantly lower in cases than controls ( $p < .000$ ) (Table 1). Numbers of NK cells were not different between CFS and controls. The values for CD3-CD56+ lymphocytes/cumm (expressed as median (25<sup>th</sup>-75<sup>th</sup> percentile) were: 176 (134-256) for CFS and 236 (151-336) for controls. According to the nonparametric ROC curve for 406 samples, as shown in Figure 1, NKCC was a good predictor of CFS status. Smaller values for NKCC indicated evidence for a positive actual state (CFS). The area under the curve (AOC) is shown in Table 2. The coordinates of the curve (COC) are given in Table S1.

### Dipeptidyl peptidase IV/CD26

We measured this peptidase on cell surfaces and in serum in a subset of samples for which we had assayed NKCC. The results shown in Table 1, with CFS compared to controls, indicated an



**Figure 1. ROC analyses were used to evaluate NKCC as a predictor of CFS.** The nonparametric ROC plot (blue curve) indicated the ability of NKCC to discriminate between CFS cases and healthy controls. Smaller values for NKCC were associated with CFS cases. The 45 degree line (green) indicates the theoretical plot of a test with no discrimination between CFS and controls.

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elevation of the percent of CD26+ CD2+ lymphocytes, but a decrease in the number of molecules of CD26 on T cells and NK cells and a decrease in the soluble form of CD26 in serum. ROC curve analyses and AUC, shown in Table 2 and Figures 2, 3, and 4 indicated that all three measures of CD26 have potential as biomarkers for CFS (see COCs in Tables S2, S3, and S4). The qualitative flow cytometry assay for proportion of CD26+CD2+ lymphocytes and the ELISA assay of sCD26 in serum were good predictors. The quantitative flow method for concentration of CD26 on CD2+ lymphocytes was less precise. Spearman analyses showed that none of the CD26 assays were significantly correlated with NKCC (data not shown).

## Discussion

Data from this and earlier studies gave credible support to diminished NKCC function in CFS. These effector cells of the innate immune system have an important role in antiviral, antibacterial, and antitumor immunity, but were deficient as measured by direct cytolysis of target cells, and as determined by measurement of intra cellular lytic proteins [14,20]. In 60 to 80% of published samples, CFS presented with acute onset of illness, with systemic symptoms similar to influenza infection that did not subside [14]. The sudden onset, the symptoms of myalgia, arthralgia, sore throat and tender lymphadenopathy prompted a theory of infection induced illness [14,27]. Published reports both support and deny associated microbial infections, reactivation of latent herpes virus infections and/or retrovirus infections in CFS [28-35]. Of interest is the finding by Glaser and colleagues that the adverse immunologic effects of persistent infections with Epstein Barr Virus (EBV) did not require viral DNA synthesis [36]. Some published work suggested the possibility of elevated risk for cancer in patients with CFS [10-11], though to date there

**Table 2.** ROC curve analysis: Area Under the Curve (AUC) for natural killer cell cytotoxicity and dipeptidyl peptidase IV/CD26 in chronic fatigue syndrome cases compared to controls.

Variables	Area	Std. Error <sup>a</sup>	Asymptotic Sig. <sup>b</sup>	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
NKCC%	.776	.024	.000	.729	.823
CD2+CD26+%	.746	.037	.000	.674	.818
sCD26 ng/ml	.732	.036	.000	.652	.794
rMolCD26/CD2+ cell	.650	.042	.001	.568	.733

<sup>a</sup>Under the nonparametric assumption;

<sup>b</sup>Null hypothesis: true area = 0.5.

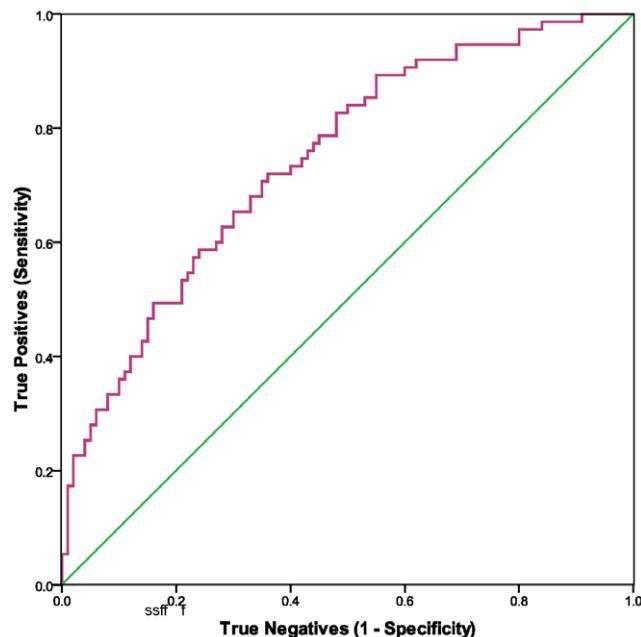
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has been no long term natural history study to accurately assess this risk.

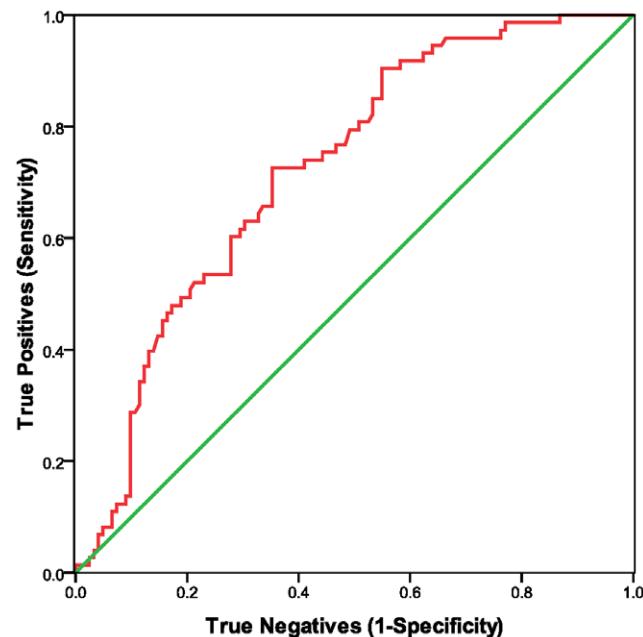
Previously, we showed that the proportion of lymphocytes (NK cells and T cells) expressing CD26 is elevated in CFS cases [14]. In the present study, we found the density of DPPIV/CD26 on lymphocyte surfaces and the concentration of the enzyme in plasma is reduced in CFS subjects, compared to controls. We hypothesize that this reduction is due to chronic lymphocyte activation in CFS patients. The present study adds to the evidence of loss of innate immune function and chronic immune activation, resulting from the long term presence of antigenic stimulus, either self or foreign. Compared to healthy controls, chronic hepatitis C patients had significantly lower serum soluble CD26 levels [37]. In another study, acute, self-limiting infection with live influenza vaccine and chronic infection with persistent antigen, such as with

cytomegalovirus (CMV), EBV or human immunodeficiency virus (HIV), was compared using multi-parameter flow cytometry and tetramer technology. These analyses identified a unique pattern of high density DPPIV/CD26 expression among influenza-specific CD8 T cells, but not among CD8 T cells specific for CMV, EBV (three different epitopes) or HIV [38]. These findings were interpreted as indicating that expression of CD26 (high) is characteristic of a memory cell, present in acute infection but not in chronic infection.

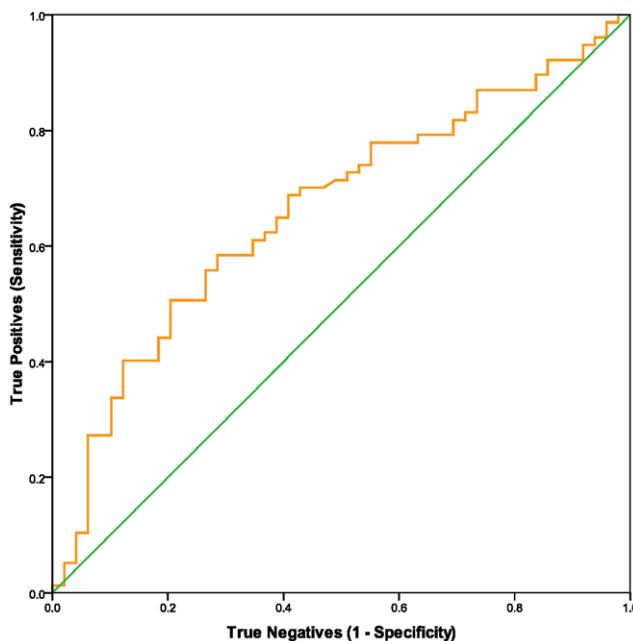
Dipeptidyl peptidase IV/CD26 cleaves N-terminal X-Pro dipeptides from peptides. The peptidase controls the *in vivo* half-life of the proinflammatory chemokine stromal cell-derived factor-1 (SDF-1). Mice deficient in DPPIV/CD26 exhibited increased levels of circulating active SDF-1, associated with increased numbers of SDF-1 receptor (CXCR4)-positive cells infiltrating



**Figure 2. ROC analyses were used to evaluate %CD26+CD2+ lymphocytes as a predictor of CFS.** The nonparametric ROC plot (purple curve) indicated the ability of %CD26+CD2+ lymphocytes to discriminate between CFS cases and healthy controls. Larger values for %CD26+CD2+ lymphocytes were associated with CFS cases. The 45 degree line (green) indicates the theoretical plot of a test with no discrimination between CFS and controls.  
doi:10.1371/journal.pone.0010817.g002



**Figure 3. ROC analyses were used to evaluate serum dipeptidyl peptidase IV/CD26 as a predictor of CFS.** The nonparametric ROC plot (red curve) indicated the ability of serum dipeptidyl peptidase IV/CD26 to discriminate between CFS cases and healthy controls. Smaller values were associated with CFS cases. The 45 degree line (green) indicates the theoretical plot of a test with no discrimination between CFS and controls.  
doi:10.1371/journal.pone.0010817.g003



**Figure 4. ROC analyses were used to evaluate relative number of molecules of dipeptidyl peptidase IV/CD26 on the surface of CD2+ lymphocytes as a predictor of CFS.** The nonparametric ROC plot (orange curve) indicated the ability of number of molecules of dipeptidyl peptidase IV/CD26 on the surface of CD2+ lymphocytes to discriminate between CFS cases and healthy controls. Smaller values were associated with CFS cases. The 45 degree line (green) indicates the theoretical plot of a test with no discrimination between CFS and controls. cell at saturating concentrations of antibody; rMol/cell) is shown.

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arthritic joints [39]. In a clinical study, by the same researchers, plasma levels of DPPIV/CD26 from rheumatoid arthritis patients were significantly decreased when compared to those from osteoarthritis patients and inversely correlated with C-reactive protein levels. They postulated that decreased circulating soluble DPPIV/CD26 levels in arthritis may influence DPPIV/CD26-mediated regulation of the chemotactic SDF-1/CXCR4 axis. These patients have elevated number of T cells expressing DPPIV/CD26 and reduced DPPIV enzymatic activity and DPPIV/CD26 antigen in plasma compared to controls [39,40].

Dipeptidyl peptidase IV/CD26 causes the degradation of glucagon-like peptide 1 (GLP-1), an incretin hormone [41]. Inhibitors of DPPIV/CD26 such as sitagliptin, which prevent the degradation of GLP-1 [42], are now marketed for the treatment of type 2 diabetes mellitus (T2DM). Considering that DPPIV/CD26 has a key role in immune regulation as a T cell activation molecule and in immune-mediated disorders, it is noteworthy that the effects of inhibition of DPPIV/CD26 on the immune system have not been extensively investigated. There are reports that infections were increased after sitagliptin treatment [43]. So far, only routine laboratory safety variables have been measured in published randomized controlled trials.

Administration of DPPIV/C26 inhibitors for the treatment of T2DM patients could influence immune function, including NKCC. A study of CD26 gene knockout mice concluded that DPPIV/C26 contributes to the regulation of development, maturation and migration of CD4 T, NK and NKT cells, cytokine secretion, T cell-dependent antibody production and immunoglobulin isotype switching of B cells [44]. An initial

diagnosis of CFS would not be made in the patient with obvious T2DM. However, the frequency of development of T2DM after diagnosis of CFS is not known—nor is the effects of a DPPIV/C26 inhibitor in the CFS patient.

Duration of illness typically exceeds 10 years. Persistence may involve complex interaction of immune, autonomic and neuroendocrine regulation and remains poorly understood. It is important to recall that the associated chronic inflammation can have important consequences on energy metabolism by promoting insulin resistance [45]. This chronic inflammatory state would also support a concurrent low-grade Th1 response by inhibiting the protective effects of T regulatory cell subset via increased IL-6 expression. The decreased NKCC and the abnormal DPPIV/C26 manifestations in CFS would be compatible with the hypothesis that the immune system of affected individuals is biased towards a T- helper (Th) 2 type, or humoral immunity-oriented cytokine pattern.

The data obtained on NK cell function, immune activation and DPPIV/C26 on cell surfaces and in serum, are consistent with a viral etiology for CFS. The elevated proportion of activated CD4 and CD8 T cells and defective NKCC in CFS cases suggests that T cells are metabolically limited in performing their helper function. The abnormalities observed may have applications with other complex, chronic and poorly understood illnesses, including fibromyalgia, gulf war illness, rheumatologic disorders and multiple sclerosis—though the precise constellation of patterns observed with these biomarkers may differ in each. However, the specific panel that we have identified here are likely to be helpful as objective markers for diagnosing CFS, determining subgroups, following patients over time and as targets for therapeutic strategies. These indicators are parts of a complex and integrated system and their inter-dependency must be addressed [46]. Accordingly, we are currently engaged in mapping the network structure of neuroendocrine-immune interaction in CFS

## Limitations

Obvious limitations of this study are that each patient sample represents a single point in time. To address this, we are conducting a large longitudinal study to follow 150 subjects over 18 months. Samples are collected during times of relative symptom remission and exacerbation. Completion of the study will allow the correlation of CFS related symptoms with lymphocyte function and activation. Because CFS is a condition that affects women in disproportionate numbers, over eighty percent of the cases in the present study were female. The larger study will have sufficient power to allow a sub study of biomarker patterns in men with CFS.

## Conclusions

The predominance of evidence indicating that people with CFS have decreased function of NK cells and abnormal activation of T and NK cells was supported by this study. The purpose of the study was to determine usefulness of these measurements as biomarkers. By ROC analysis, NKCC and dipeptidyl peptidase/CD26 were identified as potential biomarkers for CFS through their demonstrated accuracy in discriminating CFS patients from healthy controls. Dipeptidyl peptidase/CD26 on lymphocytes or in serum was not correlated with NKCC, suggesting that these are non-redundant biomarkers. Current CFS treatments are directed at reducing symptom severity but no cure exists for this condition. The findings of this study give support to the concept that cause and/or the pathophysiology of CFS are related to infection. These findings may lead to therapeutic approaches. The specter of

infectious disease further emphasizes the significance of this research to public health.

## Supporting Information

**Figure S1** Illustration of technique used to convert fluorescence intensity values to median numbers of molecules PE bound per cell (relative numbers of molecules protein expressed per cell at saturating concentrations of antibody; rMol/cell).

Found at: doi:10.1371/journal.pone.0010817.s001 (0.38 MB TIF)

**Table S1** Coordinates of the Curve for NKCC.

Found at: doi:10.1371/journal.pone.0010817.s002 (0.23 MB DOC)

**Table S2** Coordinates of the ROC Curve for CD26+CD2+ Lymphocytes in CFS Compared to Controls.

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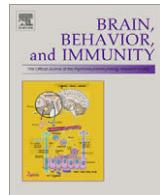
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## A formal analysis of cytokine networks in Chronic Fatigue Syndrome

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### ABSTRACT

Chronic Fatigue Syndrome (CFS) is a complex illness affecting 4 million Americans for which no characteristic lesion has been identified. Instead of searching for a deficiency in any single marker, we propose that CFS is associated with a profound imbalance in the regulation of immune function forcing a departure from standard pre-programmed responses. To identify these imbalances we apply network analysis to the co-expression of 16 cytokines in CFS subjects and healthy controls. Concentrations of IL-1 $\alpha$ , 1 $\beta$ , 2, 4, 5, 6, 8, 10, 12, 13, 15, 17 and 23, IFN- $\gamma$ , lymphotoxin- $\alpha$  (LT- $\alpha$ ) and TNF- $\alpha$  were measured in the plasma of 40 female CFS and 59 case-matched controls. Cytokine co-expression networks were constructed from the pair-wise mutual information (MI) patterns found within each subject group. These networks differed in topology significantly more than expected by chance with the CFS network being more hub-like in design. Analysis of local modularity isolated statistically distinct cytokine communities recognizable as pre-programmed immune functional components. These showed highly attenuated Th1 and Th17 immune responses in CFS. High Th2 marker expression but weak interaction patterns pointed to an established Th2 inflammatory milieu. Similarly, altered associations in CFS provided indirect evidence of diminished NK cell responsiveness to IL-12 and LT- $\alpha$  stimulus. These observations are consistent with several processes active in latent viral infection and would not have been uncovered by assessing marker expression alone. Furthermore this analysis identifies key sub-networks such as IL-2:IFN- $\gamma$ :TNF- $\alpha$  that might be targeted in restoring normal immune function.

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## 1. Background

Chronic Fatigue Syndrome (CFS) is characterized by persistent and unexplained fatigue resulting in severe impairment in daily function and is defined by symptoms, disability, and exclusion of medical and psychiatric conditions that could explain the fatigue (Fukuda et al., 1994; Reeves et al., 2003; Prins et al., 2006). The US Centers for Disease Control and Prevention (CDC) estimates that as many as 4 million people are affected with CFS in the US alone (Reeves et al., 2007; Chandler et al., 2008). Costs to the US economy are estimated at \$9.1 billion in lost productivity (Reynolds et al., 2004) and up to \$24 billion dollars in health care expenditures annually (Jason et al., 2008). Furthermore complications and co-morbidity can be severe. For example, CFS is associated with

chronic and episodic cardiovascular and autonomic dysfunction (Gerry et al., 2003). Therefore this illness has far-reaching consequences and constitutes a significant public health concern.

Evidence of chronic immune dysfunction in CFS has been reported by several groups (Klimas et al., 1990; Straus et al., 1993; Hilgers and Frank, 1994; Keller et al., 1994; Tirelli et al., 1996; Gupta et al., 1997; Patarca et al., 1997; Patarca-Montero et al., 2001; Siegel et al., 2006) though the exact nature of this dysfunction remains unclear (Maher et al., 2003). A principal avenue of investigation has been the measurement in blood of immune signals conducted by cytokines. Many of the symptoms experienced by CFS patients strongly resemble the “sickness behavior” that can be induced by the administration of pro-inflammatory cytokines. In particular decreased motor activity, altered food and water intake, sleep and cognition have been linked to increases in the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the brain (Dantzer et al., 2008). Individual cytokines however are pleiotropic and their biological activities are known to be context specific. This becomes evident when considering the current body of work focused on immune dysfunction in CFS. While some studies have reported increased levels of anti-inflammatory cytokines such as IL-10 (ter

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Wolbeek et al., 2007) and IL-4 (Skowera et al., 2004), others have shown a correlation with pro-inflammatory signals TNF- $\alpha$  and IL-6 (Gaab et al., 2005; Carlo-Stella et al., 2006). Admittedly the heterogeneity of the CFS population (Vollmer-Conna et al., 2006; Aspler et al., 2008; Kerr et al., 2008b) has been an issue. However a major failing remains analytical. In particular immunological markers continue to be analyzed individually even though their expression is articulated as part of an integrated network. In addition to the numerical advantages of a combinatorial approach, for example the control of excessive measurement noise (Szymanska et al., 2007), it is becoming apparent that understanding complex disease will require more than a list of defective cells or genes. Because cellular and molecular components are highly inter-dependent it is necessary to understand the "wiring" via which they interact (Barabási, 2007). Immune cells form a distributed network of diverse elements that exchange information through a complex web of interactions (Orosz, 2001). The architecture of such a networked system profoundly impacts its behavior (Klemm and Bornholdt, 2005) and the strategies that are available for adapting to change and maintaining homeostasis. Nonetheless, the formal analysis of biological networks in defining disease phenotypes has received relatively little attention. Recent attempts have focused on the visual comparison of relatively sparse collections of known pathway elements (Kerr et al., 2008a) or a broad description of shifts in overall structure (Emmert-Streib, 2007). We have extended this work in several important ways, introducing continuous metrics that quantify not only the degree of change but the type of change occurring in global and more importantly in local network structure. These metrics have allowed us to identify functional communities of markers within these networks as well as key elements driving disease-related changes in network structure (Fuite et al., 2008).

Here we use network constructs such as these to examine how patterns in the coordinated expression of cytokines might differ in CFS subjects. In a recent publication we introduced the multiplex method to simultaneously measure a broad spectrum of 16 cytokines in order to assess their use as biomarkers for CFS (Fletcher et al., 2009). Using this same experimental data we have now constructed separate networks describing co-expression of these 16 cytokines in a group of CFS subjects and in a group of healthy controls, respectively. Pair-wise mutual information (MI), estimated from the biological variability within each group, was used as a robust measure of association between cytokines. These networks were then analyzed using quantitative metrics rooted in graph theory to assess the importance and nature of architectural changes related to illness. In particular we assessed local changes in the degree of connectivity at cytokine nodes and the redistribution of these connections as they form distinct and more locally centered communities. Consistent with our previous work (Fuite et al., 2008) we found that these cytokine networks differed significantly in architecture between diagnostic groups emphasizing that the organizational attributes of the immune response in addition to the activation level of individual markers constitute a unique characteristic of CFS. Of note distinct modules emerged in both healthy control and CFS networks that were recognizable as components of Th1, Th2 and Th17 responses. In CFS we found consistent but significantly attenuated patterns of Th1 and Th17 response occurring in the context of a well-established Th2 inflammatory environment. These patterns would have escaped detection had the analysis focused solely on differential expression of individual cytokines. Interestingly the cytokine co-expression patterns described in this study, though not uniquely assignable to a viral pathology, were at least consistent with the disruptive effects of latent viral infection by pathogens such as Epstein-Barr virus (EBV) (Samanta and Takada, 2009; Tsuge et al., 2001).

## 2. Materials and methods

### 2.1. Sample collection and processing

#### 2.1.1. Subject cohort

Female CFS patients ( $n = 40$ ; mean age 50) were from the CFS and Related Disorders Clinic at the University of Miami. A diagnosis of CFS was made using the International Case Definition (Fukuda et al., 1994; Reeves et al., 2003). Healthy female controls ( $n = 59$ ; mean age 53) were from a NIH funded study. All CFS study subjects had a SF-36 summary physical score (PCS) below the 50th percentile, based on population norms. Exclusion criteria for CFS included all of those listed in the current Centers for Disease Control (CDC) CFS case definition, including the listed psychiatric exclusions, as clarified in the International CFS Working Group (Reeves et al., 2003). All CFS subjects were assessed for psychiatric diagnosis at the time of recruitment with the Composite International Diagnostic Instrument (World Health Organization, 1997). Based on this assessment, we excluded subjects with DSM IV diagnoses for psychotic or melancholic depression, panic attacks, substance dependency, or psychoses as well as any subjects currently suicidal. We also excluded subjects with Borderline or Antisocial Personality Disorder. Subjects had no history of heart disease, COPD, malignancy, or other systemic disorders that would be exclusionary, as clarified by Reeves et al. (2003). Subjects were excluded for the following reasons: less than 18 yrs of age, active smoking or alcohol history, history of significant inability to keep scheduled clinic appointments in past.

**Ethics statement.** All subjects signed an informed consent approved by the Institutional Review Board of the University of Miami. Ethics review and approval for data analysis was also obtained by the IRB of the University of Alberta.

#### 2.1.2. Cytokine profiles

Morning blood samples were collected into ethylene diamine tetra acetic acid. Plasma was separated within 2 h of collection and stored at  $-80^{\circ}\text{C}$  until assayed. We measured 16 cytokines in plasma using Quansys reagents and instrument (Quansys Biosciences, Logan, Utah). The Quansys Imager, driven by an 8.4 mega-pixel Canon 20D digital SLR camera, supports 96 well plate based chemiluminescent imaging. The Q-Plex™ Human Cytokine - Screen (16-plex) is a quantitative enzyme-linked immunoabsorbent assay (ELISA)-based test where sixteen distinct capture antibodies have been absorbed to each well of a 96-well plate in a defined array. Manipulation of the range of the standard curves and exposure time allowed reliable comparisons between CFS patients and controls of both low and high level cytokine concentrations in plasma. For the standard curves, we used the second order ( $k = 2$ ) polynomial regression model (parabolic curve):  $Y_p = b_0 + b_1 X_1 + b_2 X_2 \dots + b_k X_k$ , where  $Y_p$  is the predicted outcome value for the polynomial model with regression coefficients  $b_1$  to  $k$  for each degree and  $y$  intercept  $b_0$ . Quadruplicate determinations were made, i.e., each sample was run in duplicate in two separate assays. Statistics reported in Table S3 show an average coefficient of variability (CV) of 0.20 for inter-assay and 0.09 for intra-assay repeatability. Also reported in Table S3 are the lower limits of detection (LLD) for each cytokine estimated from the standard calibration curve. In many cases the standard curve yielded a negative intercept value indicating that the modified assay produced a background optical signal at zero concentration. Accordingly, the standard curves were truncated at this baseline optical intensity and no negative concentration values were estimated or used in this analysis. In the case of cytokines with positive intercept values very few samples produced results below the LLD with the exception of IL-17. While the LLD for IL-17 was lower with the modified protocol roughly

one quarter of the CFS patients, and 1 in 10 control subjects, registered average expression values below detection.

## 2.2. Statistical analysis

Association networks were constructed using mutual information criteria (MI) implemented in the ARACNe software (Margolin et al., 2006a,b). The mutual information  $MI(X;Y)$  shared by  $X$  and  $Y$  corresponds to the total entropy  $H(X)$  and  $H(Y)$  of these variables minus their joint entropy  $H(X,Y)$  (Eqs. (1)–(3)). In order to use this metric the continuous scale for the concentration of each cytokine was divided into bins defined by a set of Gaussian kernels. The optimal choice of kernel width is dependent on the sample size and the distribution statistics of the data. The algorithm used by the ARACNe platform is based on a computationally efficient estimation algorithm (Beirlant et al., 1997) and described in detail in Margolin et al. (2006a) and the Supplementary Technical Report in Margolin et al. (2006b). The null probability of each MI value was computed by sub-sampling with replacement. Subsets of 30 observations were repeatedly constructed by sampling each subject group separately. Samples were not removed from the candidate list if selected thereby making them available again for the next iteration. The final aggregate networks for each diagnostic group were generated from a consensus of 300 sub-sampled networks. Networks were stable in size over a wide range of MI significance thresholds (Supplementary Figure S1) and  $p \leq 0.001$  was used in all subsequent computations. This was used as the threshold for MI confidence in all subsequent computations. This consensus averaging across sub-sampled data sets and the fact that MI assigns equal influence to each measured value makes this approach quite robust to outliers (Craddock et al., 2006; Butte and Kohane, 2000). Nonetheless for additional detail we have included the values for conventional Spearman rank-based cross-correlation of cytokines in Tables S4 and S5 for the healthy controls (HC) and CFS patient groups, respectively.

$$H(X) = - \sum_{i=1}^n p(x_i) \log(p(x_i)) \quad (1)$$

$$H(X, Y) = - \sum_{j=1}^n \sum_{k=1}^m p(x_i, y_k) \log(p(x_i, y_k)) \quad (2)$$

$$MI(X; Y) = H(X) + H(Y) - H(X, Y) \quad (3)$$

Indirect associations were removed using data processing inequality (DPI) (Cover and Thomas, 2006). DPI states that if  $X$  and  $Z$  interact only through a third variable  $Y$  then Eq. (4) applies. Thus the smallest MI value can only come from indirect interaction. ARACNe removes this edge.

$$MI(X, Z) \leq \min[MI(X, Y); I(Y, Z)] \quad (4)$$

Topological differences in networks were evaluated using a weighted graph edit distance (Bunke, 2000) corresponding to the minimum summed “cost” associated with the removal and insertion of edges transforming one graph into the other (Dickinson et al., 2004; Harper et al., 2004). Herein we make the costs of these edit operations directly proportional to the changes in edge MI. The weighted graph edit distance,  $d_{GED}$ , between two undirected networks of order  $N$  with adjacency matrices,  $A$  and  $B$ , is computed as follows where  $a_{ij} = MI_{ij}$  if  $P(MI_{ij} > 0) \geq 0.001$ , else  $a_{ij} = 0$  and similarly for  $b_{ij}$ :

$$d_{GED} = \sum_{i=1}^N \sum_{j=1}^N |a_{ij} - b_{ij}| \quad (5)$$

Significance of this edit distance was estimated (i) using reference networks generated by random sub-sampling of HC subjects, (ii) from equal-sized random networks conserving edge weight distribution (Milo et al., 2004) and (iii) through multi-graphs conserving node degree distribution (Newman, 2004b).

Node degree centrality or direct connectivity of each node  $i$  to its immediate neighborhood  $N_i$  was computed as  $\sum_{j \in N_i} a_{ij}$ . Eigenvector centrality  $x_i$  was also computed for each node  $i$  as a measure of that node's connectivity to its remote neighbors. For the  $i$ th node the eigenvector centrality score  $x_i$  is proportional to the sum of  $x_j$  for all nodes connected to it such that:

$$x_i \propto \sum_{j \in N_i} x_j \Rightarrow x_i = \frac{1}{\lambda} \sum_{j \in N_i} x_j = \frac{1}{\lambda} \sum_{j=1}^N a_{ij} x_j \quad (6)$$

where  $N_i$  is the neighborhood of  $i$ ,  $\lambda$  is some constant and  $N$  is the order of the network. Constraining all  $a_{ij}$  and  $x_i$  to real positive values implies, by the Perron–Frobenius theorem, that only the largest principal eigenvalue solution to Eq. (6) is accepted (Kleinberg, 1999). Finally we have also scaled the principal eigenvector  $X$  to adjust for network size as follows:

$$\hat{X} = \frac{\sqrt{2}}{\|X\|} X \quad (7)$$

where  $\hat{X}$  is the normalized principal eigenvector and  $\|X\|$  is the norm. This scaling is based on a maximum of  $x_i = 1$  for the center node of a star network (Ruhnau, 2000). The two node centralities, degree and eigenvector, are among the common numerical values that measure network connectedness to imply node reach, control, and influence within groups.

The overall degree of centralization for any network of order  $N$  and normalized principal eigenvector  $\hat{X}$  is the centrality index  $C$ :

$$C_{\text{eigenvector}} = \frac{\sum_{i=1}^N (x_{\max} - x_i)}{\sum_{i=1}^N (1 - x_i)} \in [0, 1]. \quad (8)$$

Modularity,  $Q$ , is a measure of community structure within a network (Girvan and Newman, 2002; Newman, 2004a),  $Q = (\text{fraction of edges within modules}) - (\text{fraction of edges expected within modules})$ , such that (Newman and Girvan, 2004),

$$Q = \frac{1}{2m} \sum_{i,j=1}^n (A_{ij} - P_{ij}) \partial_{g_i, g_j} \in [-1, 1] \quad (9)$$

where  $m$  is the graph size,

$$m = \frac{1}{2} \sum_{i,j=1}^n A_{ij} \quad (10)$$

$n$  is the graph order,  $A_{ij}$  is a component of the symmetric weighted adjacency matrix describing the network, and  $g_i$  is the community to which node  $i$  is a member. The expected probability an edge randomly falls between two nodes is

$$P_{ij} = \frac{k_i k_j}{2m} \quad (11)$$

where  $k_i = \sum_{j=1}^n A_{ij}$  is the degree of node  $i$ .

To split any network or sub-network on the basis of maximizing modularity, a modularity matrix,  $B$ , is established having elements (Newman, 2006a),

$$B_{ij} = A_{ij} - P_{ij} \quad (12)$$

Elements of the leading eigenvector of the modularity matrix are used to direct a splitting of the network into two modules and to assign corresponding node membership based on sign (+/–) and magnitude (Newman, 2006b). This process was iterated

and modules sequentially identified until a maximum modularity for the overall network was reached or until further cuts increased modularity insignificantly ( $p > 0.05$ ).

Graphical rendering was performed using a “spring-electrical” embedding (Pemmaraju and Skiena, 2003) where nodes are idealized as similarly charged objects that repel each other. Edges are imagined as springs adhering to Hooke's law with spring-constants proportional to their MI weights. The network is relaxed iteratively to a minimum energy embedding, which naturally reveals modular structure.

### 3. Results

#### 3.1. Cytokines undergo widespread differential expression in CFS

Results of the nonparametric Wilcoxon rank-sum test comparing the difference in median expression for each cytokine in CFS versus healthy control (HC) have been presented previously (Fletcher et al., 2009) and are summarized in *Supplemental* Table S1. Briefly these show that 10 of the 16 of the cytokines surveyed had significantly different median expression levels ( $p \leq 0.05$ ) across groups. Circulating concentrations of interleukins (IL) IL-1a, 1b ( $p \leq 0.05$ ) as well as 4, 5, 6, 12 and lymphotxin-alpha (LT- $\alpha$ ) ( $p \leq 0.01$ ) were markedly higher. Conversely, CFS patients exhibited lower expression of IL-8, 13, and 15 ( $p \leq 0.01$ ). Levels of IL-2, 10, 17, and 23, interferon-gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ) showed little difference in expression between groups. Increased levels of IL-1b and IL-6 in CFS align with experimental results showing the induction of “sickness behavior” from increased levels of pro-inflammatory cytokines (Dantzer et al., 2008) in the brain.

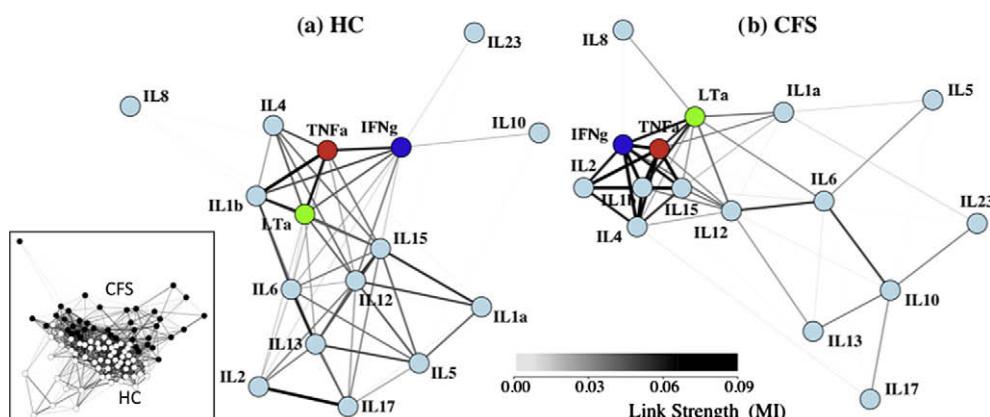
#### 3.2. Altered associations are pervasive among cytokines in CFS

In order to verify the relative homogeneity of subject groups with regard to their cytokine signatures we first used a transpose of the experimental data to construct an analogous MI association network where each subject was represented by a node. The topology of the resulting network, when viewed as a low energy embedding, showed a natural separation of subjects into two non-overlapping regions consistent with the diagnostic assignment (Fig. 1 inset). As a result all subjects in each group were used in the construction of the cytokine co-expression networks for CFS and HC, respectively. Individual networks were then constructed

for HC and CFS subjects using the within-group variability to estimate the pair-wise MI or shared information linking the expression of these 16 cytokines (Fig. 1). Random sub-sampling of the subject groups was conducted to establish confidence intervals for the graph edit distance between phenotypes (Figure S1). The narrow distribution of edit distance values separating within-group networks further supported the assessment that each diagnostic group was relative homogeneous in composition.

Summary statistics describing basic properties of the CFS and HC networks are shown in Table 1. Interestingly while the average number of links per node differed between networks the overall mutual information supported by these connections did not. An average node was connected to its neighbors by one additional link in the HC network, namely 5.9 versus 5.1 links in the CFS network ( $p < 0.01$ ). Nonetheless the mutual information carried to the average node by these connections was essentially the same if we compare the cumulated link weight of 0.236 in HC to that of 0.240 in CFS ( $p \gg 0.05$ ). Although these networks were similar in terms of their overall mutual information (cumulated edge weight) or network size, they differed significantly in how this mutual information was distributed. The CFS network had a significantly higher centrality index, 0.448 versus 0.331 in HC ( $p \ll 0.01$ ), suggesting a greater reliance on a minority of highly connected hubs. Accordingly a quantitative comparison of overall network topology showed that HC and CFS networks were separated by a weighted edit distance of  $\sim 1.96$  (Eq. (5)) as a result of this re-structuring (Figure S1). This corresponded to more than 10 standard deviations (0.13) above the expected distance between two networks constructed from randomly sampled HC subjects ( $d_{\text{edit}} \sim 0.18$ ) and 3 standard deviations ( $\sim 0.50$ ) above the expected separation between two randomly assembled multi-graphs ( $d_{\text{edit}} \sim 0.88$ ) conserving node degree (data not shown).

The spring-mass representations shown in Fig. 1 confirm that these networks were visibly different in topology. This increase in overall network centrality in CFS was driven primarily by a few interacting markers. Local re-structuring was described by changes in node degree centrality, a measure of direct connectivity, and eigenvector centrality, a combined measure of direct and indirect connectivity. Results presented in Fig. 2 indicate that nodes representing IL-1b, 2, 4, IFN- $\gamma$  and TNF- $\alpha$  concentrations became better integrated into the core network of CFS, both in terms of their association with direct and remote neighbors. Despite maintaining similar eigenvector centrality in both networks, the strength of direct connections from neighboring nodes to IL-10 substantially increased (degree centrality) in CFS. In addition,



**Fig. 1.** Networks for HC and CFS have visibly different topologies. A weighted spring-electrical embedding structurally reveals the subject–subject (inset) and cytokine–cytokine associations based on measurements in 59 healthy control subjects (a) and 40 CFS patients (b). All edge weights are significant at  $p \leq 0.01$ . Separation of subjects was consistent with their assignment to diagnostic groups supporting the use of within-group variation in the estimation of mutual information for cytokine–cytokine associations.

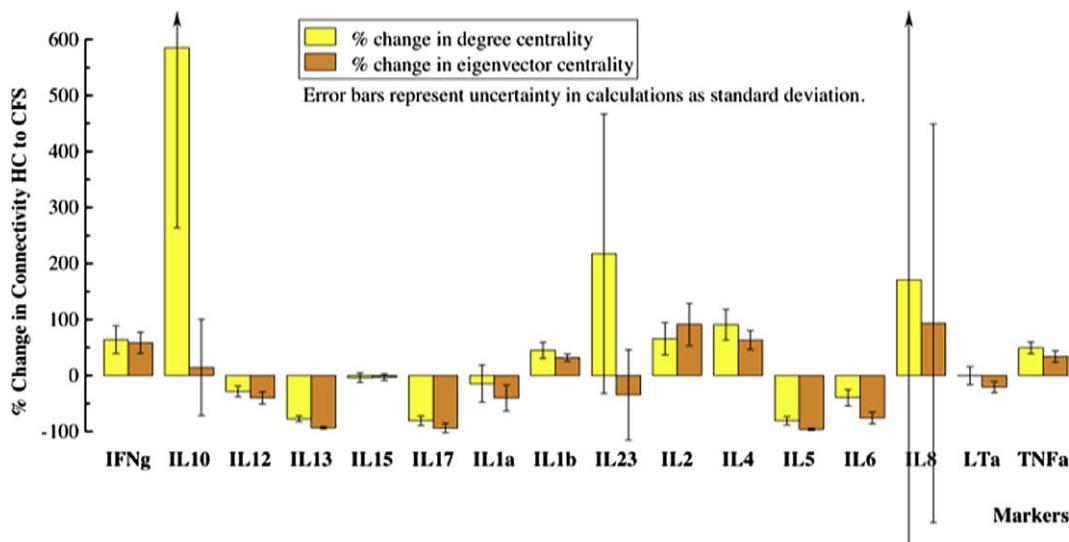
**Table 1**

Connectivity patterns differ significantly between groups. Summary of network-wide descriptive metrics with associated standard error (s) for the HC and CFS networks as well as for sub-networks I+ and II–.

Network	Metric	HC	CFS	p-Value
Overall network	Order (total number of nodes)	16	16	
	Mean links per node <sup>a</sup>	5.9 (0.2)	5.1 (0.2)	0.009
	Mean node degree <sup>b</sup>	0.236 (0.007)	0.240 (0.007)	0.689
	Centrality index	0.331 (0.011)	0.448 (0.006)	0.000
	Modularity index	0.398 (0.019)	0.394 (0.020)	0.978
Cluster I+	Order (total number of nodes)	8	10	
	Mean links per node	5.3 (0.2)	2.6 (0.2)	0.000
	Mean node degree	0.217 (0.008)	0.088 (0.005)	0.000
	Centrality index	0.187 (0.011)	0.609 (0.016)	0.000
Cluster II–	Order (total number of nodes)	8	6	
	Mean links per node	2.8 (0.2)	5.0 (0.0)	0.000
	Mean node degree	0.121 (0.005)	0.332 (0.002)	0.000
	Centrality index	0.562 (0.012)	0.112 (0.002)	0.000

<sup>a</sup> Mean links per node counts all links with non-zero weight as 1 link.

<sup>b</sup> Mean node degree uses the link weight or MI value.



**Fig. 2.** Most cytokines significantly modified their connectivity in the CFS state. These network alterations were revealed by the relative change in the total weight of edges connected at each node (node degree centrality) as well as edges acquired through first neighbors (normalized eigenvector centrality). Interleukins (IL), 2, 4, and 1b, interferon-gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ) became much better integrated into the core network in CFS, while interleukins, 5, 6, 12, 13, and 17 became more weakly associated.

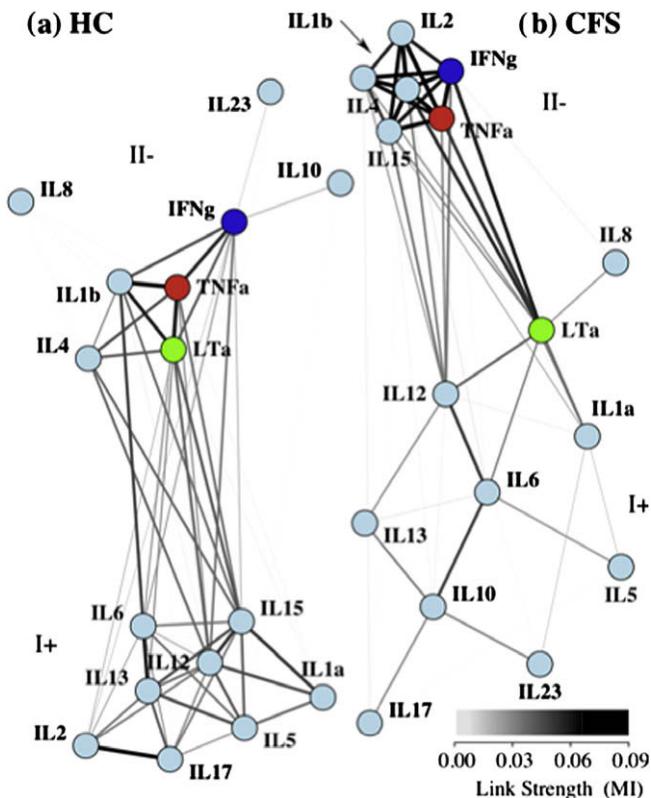
IL-10 shifted from having a weak association to a core node (IFN- $\gamma$ ) in HC to having stronger associations to an opposite group of nodes in CFS (IL-6, 13, 17, 23) (Figs. 2 and 3). Markers that were much less strongly connected in CFS were IL-5, 6, 12, 13, and 17 (Table S1). By the same token cytokines IL-8, 15, and 23 remained unchanged in their degree of overall integration in the CFS and HC networks.

### 3.3. Mid-scale shifts in network structure

The distribution of connections in each network among sets of nodes suggested that both the HC and CFS networks were made up of sub-networks. To analyze the extent of community structure within each network we iteratively divided the set of cytokine nodes into subsets and calculated increase in overall network modularity. Results indicated that the extent of community structure in the HC and CFS networks was about the same with maximal modularity values of 0.398 and 0.394, respectively. These values were achieved when the networks were broken down into two component modules, labeled I+ and II– (Table 1, Fig. 3). Separation into additional modules either lead to a decrease in modularity, or

did not significantly increase the modularity index at  $p < 0.05$  confidence.

Results in Table 1 show that although both HC and CFS networks were made up of two mid-scale communities; these constituent modules possessed important differences in internal structure. Cluster I+ became less densely linked in CFS patients as measured by a significant decline in number and strength of internal node associations. In cluster I+ of the CFS network the mean number of links per node fell from 5.3 to 2.6 ( $p < 0.01$ ) and the mean node degree fell from 0.217 to 0.088 ( $p < 0.01$ ). In addition cluster I+ became structurally more hub-like in CFS with an increase in centrality index to 0.609 from 0.187 in HC ( $p < 0.001$ ). Conversely cluster II– became structurally less focused in CFS dropping in centrality index from 0.562 to 0.112 ( $p < 0.001$ ). More evenly connected, cluster II– was also more densely linked in CFS patients with significant increases in the number and strength of internal node associations. The mean number of links per node rose from 2.8 to 5.0 ( $p < 0.01$ ) in cluster II–, and the mean node degree increased from 0.121 to 0.332 ( $p < 0.01$ ) in the CFS network.



**Fig. 3.** Both HC and CFS networks are composed of two distinct communities. Visually “relaxing” the links between identified communities of nodes and allowing them to drift apart emphasizes community structure in both networks. Overall modularity was maximized when each network was separated into two communities with differing compositions, labeled I+ at the top and II- at the bottom. Each community represents a clustering of nodes with a greater internal linkage than would be expected compared to a random sampling of similar nodes.

**Table 2**

Cytokines change community membership in CFS. Membership score with standard error (±) to either of the two modules, I+ or II- for each cytokine node in HC and CFS networks. The magnitude of the membership score indicates the strength with which nodes are associated to the module they belong. Change in modularity membership score tracks differences in community association for each marker.

Marker	Module membership		Change in membership	p-value
	HC	CFS		
IL-1a	0.17 (0.09)	0.04 (0.06)	-0.13 (0.15)	0.004
IL-1b	-0.34 (0.04)	-0.28 (0.04)	0.06 (0.07)	0.002
IL-2	0.24 (0.06)	-0.36 (0.02)	-0.60 (0.07)	0.000
IL-4	-0.24 (0.03)	-0.29 (0.04)	-0.05 (0.07)	0.010
IL-5	0.33 (0.04)	0.13 (0.03)	-0.19 (0.07)	0.000
IL-6	0.11 (0.09)	0.41 (0.03)	0.30 (0.11)	0.000
IL-8	-0.07 (0.09)	0.02 (0.01)	0.10 (0.10)	0.176
IL-10	-0.04 (0.01)	0.41 (0.04)	0.46 (0.05)	0.000
IL-12	0.13 (0.06)	0.17 (0.05)	0.04 (0.11)	0.199
IL-13	0.28 (0.03)	0.23 (0.05)	-0.05 (0.09)	0.028
IL-15	0.05 (0.04)	-0.24 (0.03)	-0.30 (0.07)	0.000
IL-17	0.36 (0.03)	0.12 (0.03)	-0.24 (0.06)	0.000
IL-23	-0.03 (0.04)	0.15 (0.03)	0.17 (0.06)	0.000
IFN- $\gamma$	-0.29 (0.03)	-0.26 (0.03)	0.03 (0.07)	0.082
LT- $\alpha$	-0.30 (0.04)	0.03 (0.04)	0.33 (0.08)	0.000
TNF- $\alpha$	-0.42 (0.02)	-0.29 (0.01)	0.13 (0.03)	0.000

In addition to changes in structure we also observed changes in the composition of modules. The membership of an individual node to its respective module was measured by its centrality within the modularity matrix. This shifted significantly in CFS as a result of changing pair-wise associations (Table 2). In CFS the

markers IL-10, IL-23 and LT- $\alpha$  shifted from cluster II- to cluster I+. While IL-6 strengthened its position in cluster I+ of the CFS network it shed the direct and strong association it held with IL-1b in HC. Conversely the markers, IL-2 and IL-15 moved in the opposite direction, significantly shifting centrality away from cluster I+ and towards cluster II- in CFS. These changes in centrality were significant at  $p < 0.001$ . In contrast IL-8 maintained marginal association with either of these node communities in both CFS and HC.

#### 4. Discussion

In order to explore changes in the patterns of immune activity in CFS we constructed two distinct association networks linking the expression of 16 cytokines measured in plasma for 40 female patients and 59 case-matched healthy controls (HC). Quantitative analysis of these two networks indicated that their topologies differed far beyond what would be expected by chance alone. Indeed variation separating the patterns of cytokine-cytokine association from each subject group was 10 times greater than the variability found within each group. Interestingly the average cytokine node in either network supported the same overall exchange of mutual information. This being said a typical CFS network node relied on one less connection to do so. This is an important point as it suggests that despite differences in cytokine expression between groups both networks were equally coherent overall ( $p = 0.689$ , Table 1). Even at the basal levels of cytokine expression found in the HC group the correlation linking cytokines into a network was not only significant (all edges  $p < 0.001$ ) but it was virtually equivalent to the overall strength of association supporting the CFS network. Instead the difference between CFS and HC networks arose from a redistribution in the routing of mutual information with the CFS network relying more strongly on a minority of highly connected hubs. Driving these changes in structure we found that cytokines IL-1b, 2, 4, IFN- $\gamma$ , TNF- $\alpha$  became much better integrated into the core CFS network, so much so that these formed a distinct sub-network. Direct connections to anti-inflammatory cytokine IL-10 also increased substantially in CFS while the reverse was true of IL-13, 17 as well as IL-5 and 6. Despite this local re-structuring these very different cytokine networks still shared a similar overall granularity. Using a novel measure of modularity we dissected these cytokine networks and found that two mid-scale communities could be isolated in both the CFS and HC group: clusters I+ and II-. However a closer look at the internal structure of these communities revealed diametrically opposite designs across illness groups. In CFS cytokine nodes in cluster I+ were more sparsely connected and adopted a more hub-like architecture whereas cytokine nodes in cluster II- were more strongly and more uniformly interconnected. The exact opposite is true of these same clusters in the control network. Differences such as these reinforce the notion that CFS manifests not only as a difference in the expression level of individual cytokines but also as an important shift in the patterns of association linking these cytokines.

The emergence of a tight-knit cluster dominated by Th1 cytokines was perhaps the most significant and most visible feature of the CFS network. Consisting of cytokine nodes IL-1b, IL-4, IFN- $\gamma$  and TNF- $\alpha$  cluster II- also saw the recruitment of cytokines IL-2 and IL-15 from their position in cluster I+ of the HC network. This group became much more tightly associated in CFS and less centered about any individual cytokine. Interestingly IL-2, 4 and 15 belong to a family of cytokines that also includes IL-7, IL-9 and IL-21. Members of this family share a receptor complex consisting of IL-2 specific IL-2 receptor alpha (CD25), IL-2 receptor beta (CD122) and a common gamma chain ( $\gamma$ c). It is not surprising therefore to observe a strong association between these network nodes upon immune activation. IL-2 and IL-4 are both T cell growth

factors though the latter is a much more effective promoter of B cell proliferation (Burke et al., 1997). In these data, the IL-4 median concentration was increased 3-fold in CFS while IL-2, IFN- $\gamma$  and TNF- $\alpha$  concentrations remained unchanged. This would support the presence of an active Th2 component in CFS and an antagonistic role for IL-4 towards Th1 cytokines such as IFN- $\gamma$  within cluster II-. Additionally new recruits, IL-2 and IL-15, both contribute to NK cell proliferation. Though NK cell response was not assessed directly in this work, the lower levels of IL-15 and unchanged levels of IL-2 observed here appear consistent with reports of deficient NK cell response in CFS (Maher et al., 2005).

Contrary to cluster II-, cluster I+ was dominated by cytokines typically associated with innate immunity and/or Th2 adaptive response namely IL-5, 6, 10, 12 and 13. For the most part associations between cytokine nodes in cluster I+ were fewer in number and visibly weaker than those linking their counterparts in cluster II-. Despite having weaker ties the circulating levels of IL-5, IL-6 and IL-1a were significantly elevated suggesting an established Th2 inflammatory environment. Indeed in CFS the mean node degree within cluster I+ was 4-fold lower than that of cluster II- (Table 1) and the centrality index 6-fold higher suggesting a much sparser and more centrally directed pattern of interaction. Especially recognizable in CFS cluster I+ is the relatively strong association of pro-inflammatory cytokine IL-6 with anti-inflammatory counterpart IL-10. Recall that IL-10, though not differentially expressed, shifted from having a weak association with cluster II- in the HC network to this much more central role in cluster I+ opposite IL-6 in CFS. This altered role would have gone unnoticed in a more conventional analysis. Also recognizable are elements of the IL-23/Th17/IL-17 response (Boniface et al., 2008; Aggarwal et al., 2003; McGeachy et al., 2007). The direct antagonism of IL-17 response by IL-2 (Laurence et al., 2007) observed in the HC network was absent in CFS. Instead an alternative response emerged whereby IL-17, IL-23 and IL-6 were all separated by IL-10. IL-6 typically enhances IL-1b-driven IL-17 production (Louten et al., 2009; Perona-Wright et al., 2009) while IL-10 is known to effectively down-regulate Th17 cytokine expression in macrophages and T cells (Gu et al., 2008). In these data median concentrations of IL-17 and 23 were unchanged despite elevated levels of IL-1b and IL-6. Though Th17 activation was not measured directly these observations suggest that responsiveness of this subset, like that of NK cells, may be altered in CFS.

Another key feature of the CFS network is the central role that the hub nodes LT- $\alpha$  and IL-12 (Fig. 3b) play in linking cytokine clusters I+ and II-. In contrast this role is almost evenly shared between IL-6, IL-15 and IL-2 in the HC network. No longer a member of cluster II- in CFS, the LT- $\alpha$  hub nonetheless maintains strong associations to IL-1b, TNF- $\alpha$  and IFN- $\gamma$ . Primarily a product of activated T and B-lymphocytes, LT- $\alpha$  shares a strong homology with TNF- $\alpha$  and IL-1b and is a powerful inducer of both these cytokines (Kasid et al., 1990). Moreover IFN- $\gamma$  has been shown to increase the number of receptors for TNF- $\alpha$  and LT- $\alpha$  further promoting their action (Aggarwal et al., 1985). In opposition to this, IL-4 will inhibit IL-2 triggered production of TNF- $\alpha$  and LT- $\alpha$  in mixed PBMC populations (Kasid et al., 1990). The network links identified here indicate that these known responses of IL-1b and TNF- $\alpha$  to LT- $\alpha$ , and to a lesser extent IFN- $\gamma$ , remained consistently expressed in the data. However, while the expression of IL-1b increased 2-fold in CFS, that of TNF- $\alpha$  remained unchanged despite an almost 4-fold increase in LT- $\alpha$ . This attenuated TNF- $\alpha$  response in CFS could in principle be linked with the absence of IFN- $\gamma$  engagement and the elevated levels of IL-4 (>3-fold) observed in these patients. In comparison to LT- $\alpha$ , the association of IL-12 with the nodes of cluster II- is much weaker. Typically released by macrophages and dendritic cells, IL-12 is known to stimulate the production of IFN- $\gamma$  and TNF- $\alpha$  from NK and T cells. This effect is enhanced by

IL-2 (Wang et al., 2000) and to a lesser extent by IL-4 (Bream et al., 2003), a cytokine normally suppressive of IFN- $\gamma$  production. Though elevated 2-fold in this CFS cohort, the absence of a concordant IFN- $\gamma$  response further supports a dampened sensitivity of NK cells to IL-12 signaling in CFS. This may be due at least partially to inadequate IL-2 priming of IL-12 receptor expression (Wang et al., 2000) since IL-2 concentrations remained unchanged.

Viral triggers such as EBV and human cytomegalovirus (HCMV) have long been suspected of involvement in the onset and persistence of CFS. Recent evidence of xenotropic murine leukemia virus-related virus (XMRV) involvement in CFS (Lombardi et al., 2009) further supports this hypothesis. While other causes may underlie the cytokine expression patterns observed in this work many of these are at least consistent with some of the disruptive effects of chronic viral infection. In one potential model, infection with one or several viral agents may trigger or exploit deficient responsiveness of NK cells to IL-12 and LT- $\alpha$ , both of which are actively produced by EBV-immortalized B cells (Airolidi et al., 2002; Thompson et al., 2003), leading to impaired IFN- $\gamma$  production and Th1 activation. In this scenario increased IL-6, also produced by EBV-infected B cells, together with depressed levels of IL-15 may interfere with LT- $\alpha$  and IL-12 activation of NK cells and the resulting IFN- $\gamma$  production (Wilson et al., 2001; Saghafian-Hedengren et al., 2009). It is important to note however that while many of the patterns found here aligned with known EBV processes others did not; for example the lack of elevated IL-10 (Samanta et al., 2008) and IL-13 (Tsai et al., 2009). As very distinct illnesses arise from the expression of specific subsets of the 12 known EBV induced genes (Tsuge et al., 2001) the notion that CFS may involve a form of restricted viral latency may be worthy of consideration. Finally from a methodological perspective we observed that several significant shifts in network structure involved cytokines that were not differentially expressed across subject groups. This underscores the significance of co-expression analysis in understanding complex illnesses such as CFS. In particular such an analysis makes it possible to detect low-grade immune processes that may operate consistently with relatively modest changes in marker expression.

## Authors' contributions

Conceived and designed the experiments: M.A.F., N.G.K., G.B. Performed the experiments: M.A.F., N.G.K. Analyzed the data: J.F., A.K., G.B. Contributed reagents/materials/analysis tools: M.A.F., A.K., G.B., J.F. Wrote the paper: G.B., M.A.F., J.F., A.K., S.D.V., N.G.K.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.04.012.

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